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FORMULARIO ÚNICO DE CORRECCIONES Y MODIFICACIONES

①

SOLICITUD DE:

☐ Corrección de Error Material

☒ Modificación a una solicitud

②

SOLICITANTE

Nombre: **SANOFI AVENTIS DEUTSCHLAND GMBH**

Dirección: **65926 Frankfurt am Main
Alemania**

Nacionalidad o Domicilio: **Frankfurt
Alemania**

Lugar de Constitución: **Alemania**

Teléfono:

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IDENTIFICACIÓN

C.C.

☐

NIT

☐

C.E.

☐

Otro

☐

Cual

Número

③

REPRESENTANTE
O APODERADO

Nombre: **LUIS PATIÑO LEYVA**

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IDENTIFICACIÓN

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2.930.616

TPA

2698

④

Número de Expediente:

03,077,712

⑤

Descripción de la corrección o modificación solicitada:

Presento nuevo pliego reivindicatorio con 12 reivindicaciones con el objeto de subsanar las objeciones hechas a la claridad, novedad y nivel inventivo de la solicitud.

⑥

Comprobante de pago No.

07-59,331

Fecha

23 de julio de 2007

⑦

Anexos:

☒

Comprobante de pago de la tasa.

☐

Poderes si fuere el caso, junto con la sustitución de los mismos a mi favor.

☐

Documento que acredita la existencia y representación legal de las partes, cuando sean personas jurídicas

☒

Nuevo capítulo reivindicatorio

⑧

NOMBRE

LUIS PATIÑO LEIVA

FIRMA



CC 2.930.616 de Bogotá - TPA 2698 CSJ

25 JUL 2007

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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

NIT : 000176089-2

OFICIAL DE CAJA : 07 - 59,331
FECHA : JULIO 23 DE 2007

CONSIGNACION

DEPOSITANTE	TIPO PAGO	BANCO	CUENTA	No. PAGO	FECHA PGO	Vr. PAGO
MANUEL MIRANDA	CONSIGNACION	BANCO POPULAR	050-00110-6	63907897	23/07/2007	457,000.00
JAIRO SANCHEZ	CONSIGNACION	BANCO POPULAR	050-00110-6	63907898	23/07/2007	99,000.00

CONCEPTO

NT. RENTISTICO	CONCEPTO	TOTAL CONCEPTO
1 50005-01-03 CAMBIO DE NOMINIDAD Y MODIFICA	12 MARCAS Y LENAS COMERCIALES	99,000.00
	TOTAL :	99,000.00

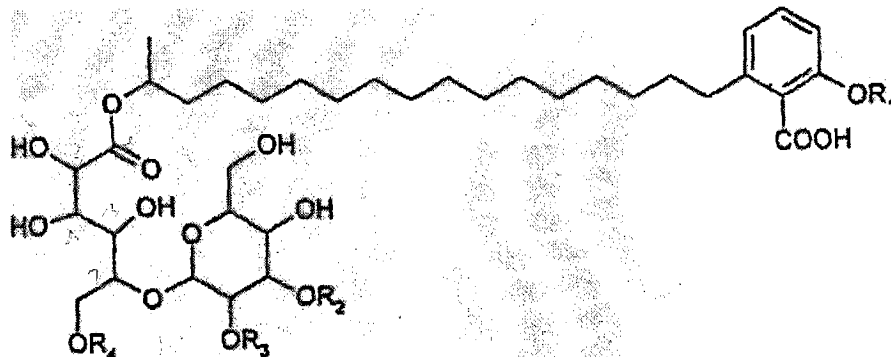
SEN: NOVENTA Y NUEVE MIL PESOS

RESPONSABLE :

RECIBO DE CAJA APLICADO AL EXPEDIENTE No.

REIVINDICACIONES MODIFICADAS

1. Un compuesto de la fórmula I



en la cual

R1, R2 y R3 independientemente uno del otro son H o radicales acilo que tienen 2-10 átomos de carbono; y
R4 es H o $-C(=O)(CH_2)_nCOOH$, en donde n es 1 a 7;
con la excepción de que R1, R2, R3 y R4 no son todos H;
y sus sales fisiológicamente toleradas

2. Un compuesto de la fórmula I de acuerdo con la reivindicación 1, en la cual R1, R2, R3 independientemente uno del otro son H o acetilo; y R4 es H o malonilo (n = 1);
y sus sales fisiológicamente toleradas.

3. Un compuesto de la fórmula I de acuerdo con la reivindicación 1 o 2, en el cual

R1 es acetilo R2, R3 y R4 son H;
y sus sales fisiológicamente toleradas.

4. Un compuesto de la fórmula I de acuerdo con la reivindicación 1 o 2, en el cual

R1 y R3 son acetilo;
R2 es H; y
R4 es malonilo;
y sus sales fisiológicamente toleradas.

5. Un compuesto de la fórmula I de acuerdo con la reivindicación 1 o 2, en el cual

R1 y R2 son H;
R3 es acetilo; y
R4 es malonilo;
y sus sales fisiológicamente toleradas.

6. Un compuesto de la fórmula I de acuerdo con la reivindicación 1 o 2, en la cual

R1 y R3 son H;
R2 es acetilo; y
R4 es malonilo;
y sus sales fisiológicamente toleradas.

7. Un compuesto de la fórmula I de acuerdo con la reivindicación 1 o 2, en la cual

R1, R2 y R4 son H; y
R3 es acetilo;
y sus sales fisiológicamente toleradas.

8. Procedimiento para la preparación de un compuesto de la fórmula I o de una sal de él fisiológicamente tolerada de acuerdo con una o varias de las reivindicaciones 1-7, caracterizado por que el microorganismo *Gloeoporus dichrous* (Fr.:Fr.) Bres. ST001714, DSM 13784, o de una de sus variantes o mutantes se fermentan bajo condiciones apropiadas, uno o varios derivados de caloprosido se aíslan y eventualmente ellos son convertidos en sales fisiológicamente toleradas.

9. Procedimiento de acuerdo con la reivindicación 9, caracterizado porque la fermentación se lleva a cabo bajo condiciones aeróbicas a una temperatura entre 18 y 35°C y a un pH entre 5 y 8.

10. Medicamento con un contenido de por lo menos un compuesto de la fórmula I o una sal de él fisiológicamente tolerada de acuerdo con una o varias de las reivindicaciones 1-7.

11. Procedimiento para la preparación de medicamentos de acuerdo con la reivindicación 11, caracterizado porque al menos un compuesto de la fórmula I o una sal de él fisiológicamente tolerada de acuerdo con una o varias de las reivindicaciones 1-7 se convierte en una forma de dosis apropiada con excipientes y/o vehículos apropiados.

12. El microorganismo *Gloeoporus dichrous* (Fr.:Fr.) Bres. ST001714, DSM 13784.

A Role for Nuclear Phospholipase C β_1 in Cell Cycle Control*

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Phosphoinositide signaling resides in the nucleus, and among the enzymes of the cycle, phospholipase C (PLC) appears as the key element both in *Saccharomyces cerevisiae* and in mammalian cells. The yeast PLC pathway produces multiple inositol polyphosphates that modulate distinct nuclear processes. The mammalian PLC β_1 , which localizes in the nucleus, is activated in insulin-like growth factor 1-mediated mitogenesis and undergoes down-regulation during murine erythroleukemia differentiation. PLC β_1 exists as two polypeptides of 150 and 140 kDa generated from a single gene by alternative RNA splicing, both of them containing in the COOH-terminal tail a cluster of lysine residues responsible for nuclear localization. These clues prompted us to try to establish the critical nuclear target(s) of PLC β_1 subtypes in the control of cell cycle progression. The results reveal that the two subtypes of PLC β_1 that localize in the nucleus induce cell cycle progression in Friend erythroleukemia cells. In fact when they are overexpressed in the nucleus, cyclin D3, along with its kinase (cdk4) but not cyclin E is overexpressed even though cells are serum-starved. As a consequence of this enforced expression, retinoblastoma protein is phosphorylated and E2F-1 transcription factor is activated as well. On the whole the results reveal a direct effect of nuclear PLC β_1 signaling in G₁ progression by means of a specific target, i.e. cyclin D3/cdk4.

It is demonstrated that an autonomous intranuclear inositolide cycle exists and that nuclear PLC β_1 ¹ is a key enzyme for cell proliferation and differentiation (1). The enzymes of polyphosphoinositide turnover occur in the nucleus of mammalian cells and yeast (Ref. references therein), and there is evidence for phosphatidylinositol bisphosphate (PIP₂) synthesis and degradation in the nuclear matrix (3). The evidence obtained with confocal and electron microscope analysis indicates that enzymes required for the synthesis and hydrolysis of phosphoi-

nositides are localized at ribonucleoprotein structures of the inner nuclear matrix involved in transcript processing within the interchromatin domains (4). Although phosphatidylinositol cycle is activated only in nuclei from HeLa cells in S phase (5), striking changes occur mainly in PLC β_1 activity a few minutes after growth factor stimulation (1). PLC β_1 is composed of two subtypes, 150-kDa PLC β_1 a and 140-kDa PLC β_1 b, that are derived from a single gene by alternative RNA splicing (6). The two forms of the PLC β_1 are detectable both in cytosolic and nuclear fractions although PLC β_1 b exists almost entirely in the nucleus (7), and the β_1 a form localizes in equal amount in nuclei and plasma membranes (8). Previous investigations from our group have demonstrated that the nucleus-confined PLC β_1 is directly involved in maintaining the undifferentiated state of Friend erythroleukemia cells even in the presence of inducers of erythroid differentiation, possibly due to a continuous stimulation of the cell cycle (9). With the above in mind, we sought to explore whether PLC β_1 is actually involved in the regulation of cell cycle machinery.

In mammalian cells, proliferation is under the control of factors that regulate the transition between different cell cycle stages at two main checkpoints. The better described checkpoint is at the G₁-S phase transition for initiation and completion of DNA replication in S phase. The other checkpoint is at the G₂-M phase transition and controls mitosis and cell division (10). The cell cycle is primarily regulated by a family of structurally related serine/threonine protein kinases, which consist of a regulatory subunit, a cyclin, and a catalytic subunit, a cyclin-dependent kinase (cdk). In mammalian cells, cdk4 or cdk6, in combination with the D type cyclins (D1, D2, and D3), and cdk2 in association with cyclin E play key roles in regulating G₁ progression (Ref. 11 and references therein).

Furthermore, overexpression of E or D type cyclins is able to accelerate the transition through the G₁ phase of the cell cycle (12). Several lines of evidence suggest that the primary function of D-type cyclins is to stimulate progression through G₁, rather than to promote the G₁/S transition. In addition it appears that in hematopoietic cell lines, overexpression of cyclin D2 or D3 prevents granulocyte differentiation, whereas overexpression of cyclin D1, which is normally not expressed in those cells, does not (13). Both cyclin D in complex with cdk4 or cdk6 and cyclin E-cdk2 phosphorylate the product of the retinoblastoma gene, the retinoblastoma protein (pRb), a well known tumor suppressor. This tumor suppressor activity relies on pRb role in gating S phase entry through its ability to repress genes activated by the E2F family of transcription factors. In fact phosphorylated pRb (ppRb) releases members of the E2F family that play an integral role in cell cycle progression by inducing the expression of gene required for S phase

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¹ The abbreviations used are: PLC, phospholipase C; IP₃, inositol polyphosphates; IGF-1, insulin-like growth factor 1; PIP₂, phosphatidylinositol bisphosphate; cdk, cyclin-dependent kinase; E₂F, retinoblastoma; pRb, retinoblastoma protein; ppRb, phosphorylated retinoblastoma protein; DTT, dithiothreitol; wt, wild type.

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entry, including those involved in DNA synthesis such as S phase regulatory factors cyclin E, cyclin A, and cdk2 (14, 15). All five E2F family members bind the same DNA sequence, but additional levels of regulation have been observed as follows: E2F-1, E2F-2, and E2F-3 associate with pRb (p107); E2F-pRb complexes are found primarily in G₁; E2F-4 and E2F-5 preferentially bind to p107 and p130. Enforced expression by microinjection of E2F-1 induces starved cells to progress through G₁ and enter S phase (16). In order to understand the mechanism that couples nuclear PLC β_1 and cell cycle machinery, we carried out a detailed analysis of the expression of the proteins involved in the regulation of the cell cycle. For this purpose we have overexpressed PLC β_1 a and -b in the nuclear compartment and the mutant M2b, which lacks the nuclear localization sequence (8, 9), in the cytoplasmic compartment in order to find out how nuclear localization of PLC β_1 acts on cell cycle progression and on key signaling events that regulate the passage through G₁ phase.

EXPERIMENTAL PROCEDURES

Construction of Expression Vectors and Transfection.—The full-length cDNA for rat PLC β_1 a (17), the full-length cDNA for rat PLC β_1 b (6), and the mutant for nuclear localization sequence, where by site-directed mutagenesis lysine residues 1056, 1063, and 1070 in region 2 of the COOH terminus were substituted with isoleucine (8), were cloned into pRc/CMV (Invitrogen) expression vector plasmid as described elsewhere (18). Friend cells were transfected using the following protocol. Briefly, a mixture of 2.5 μ g of plasmid DNA and 10 μ l of Transfectam (Promega) was added to 1×10^6 cells in 1 well of a 6-well plate for 24 h. The transformants were selected by limiting dilution in medium containing the neomycin analogue G418 at a concentration of 500 ng/ml. Clones were harvested and expanded separately in the presence of G418.

Cell Culture.—Murine erythroleukemia cells (Friend cells, clone 707) were grown in RPMI 1640 supplemented with 10% fetal calf serum. For experimental procedures cells were serum-deprived for 48 h and then stimulated with fresh serum for the indicated hours (figure legends).

Isolation of Nuclei.—A hypotonic shock combined with non-ionic detergent, essentially described by Martelli *et al.* (19), has been used. In addition to 0.5 mM phenylmethylsulfonyl fluoride, 10 mM β -mercaptoethanol, 1 mM EGTA, 10 μ g/ml leupeptin, 0.3 μ M aprotinin, 15 μ g/ml calpain I inhibitor, and 7.5 μ g/ml calpain II inhibitor were also added to the buffers. Nuclear purity was assessed by detection of β -tubulin as described in Ref. 20, and only nuclei showing in Western blot a complete absence of β -tubulin were used in the reported experiments.

Preparation of Cytoplasmic Fraction.—The cytoplasmic fraction was obtained by homogenizing cells with 20 strokes in a Dounce homogenizer in 10 mM Tris-Cl, pH 7.5, 2 mM MgCl₂ plus protease inhibitors as above and then pelleting the nuclei at 400 $\times$ g. This procedure allows the recovery of pure cytoplasmic fraction and avoids the risk of contamination by nuclear debris that is present in the crude supernatant from nuclear purification.

PLC Assay.—The assay was carried out exactly as described previously (20). Nuclear PLC activity *in vitro* has been checked by using [³H]phosphatidylinositol bisphosphate and then analyzing the nanomoles of inositol 1,4,5-trisphosphate released per mg of protein/30 min of incubation.

Flow Cytometry.—Friend cells transfected with empty vector, clones overexpressing PLC β_1 a and -b, and mutants for nuclear localization sequence were analyzed by flow cytometry. Briefly cells were grown with 10% fetal calf serum for 48 h and then starved for 24 h. Cells were washed in cold phosphate-buffered saline and fixed in cold 70% ethanol for 30 min. The pellet was resuspended in a solution containing 50 μ g/ml propidium iodide, 1 mg/ml sodium citrate, 1 μ l/ml Triton X-100 and 5 μ g/ml RNase A and analyzed by a FACStar Plus flow cytometer (Becton Dickinson).

Electrophoretic Mobility Shift Assay.—Nuclear extracts were prepared by resuspending the cells in 400 μ l of swelling buffer (10 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl), followed by incubation on ice for 10 min. Next the lysates were vortexed for 10 s before centrifugation for 1 min at 14,000 rpm at 4 $^{\circ}$ C. The pelleted nuclei were resuspended in 80 μ l of lysis buffer (20 mM HEPES, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.3 mM EDTA) and left on ice for 20 min with occasional mixing. Subsequently, the lysates were centrifuged at 14,000 rpm for 10 min at 4 $^{\circ}$ C, and the supernatant was stored at -70 $^{\circ}$ C. All

buffers were supplemented with 0.5 mM DTT and 0.2 mM phenylmethylsulfonyl fluoride. A synthetic oligonucleotide containing the consensus binding site for E2F-1, 5'-ATTAAAGTTTGGGCCCCCTTCTCAA-3' (Santa Cruz Biotechnology), was used as a probe for the assay. The probe was labeled with [³²P]dATP using T₄ polynucleotide Kinase (Promega). 10 μ g of nuclear extract was used in each binding reaction. Where appropriate, the extract was preincubated for 10 min at room temperature with 5 pM unlabeled E2F-1 oligonucleotide and 5 pM un-specific competitor. For antibody perturbation supershift experiments, 0.25 μ g of anti-pRb antibody (Santa Cruz Biotechnology) was added to the incubation 10 min prior to addition of oligonucleotide probe and incubated at room temperature. Subsequently, binding to 1 μ l of radioactive E2F-1 oligo was carried out in a reaction mixture containing 1 μ g of poly(dI-dC), 20 mM HEPES, pH 7.6, 70 mM KCl, 5 mM MgCl₂, 0.05% Nonidet P-40, 12% glycerol, 1 mg/ml bovine serum albumin, 0.5 mM DTT for 30 min at room temperature. DNA-protein complexes were separated on a 4% nondenaturing polyacrylamide gel and visualized by autoradiography.

Immunoblot Analysis.—Immunoblot analysis of 500 μ g of nuclear and cytoplasmic fractions of wt were immunoprecipitated with 2.5 μ g of anti-PLC β_1 a + b isoform, (Transduction Laboratories) for 2 h at 4 $^{\circ}$ C with constant agitation, followed by 1 h of incubation with 50 μ l of protein A-Sepharose (10% w/v). Pellets were washed three times in phosphate-buffered saline containing 1% Nonidet P-40, twice in 0.1 M Tris, 0.5 M LiCl, and twice in Tris, NaCl, EDTA (TNE). Pellets were resolved by SDS-polyacrylamide gel electrophoresis and blotted to nitrocellulose membranes for subsequent immunodetection with the same antibody, followed by detection using ECL (Amersham Pharmacia Biotech). For Western blot analysis, proteins from nuclear extract (20 μ g) were separated on an SDS-0.1% polyacrylamide gel and transferred onto nitrocellulose paper. The following antibodies were used: polyclonal antibodies to cyclin D3, cyclin E, cdk2, and cdk4 from Santa Cruz Biotechnology; polyclonal antibodies to pRb and to ppRb for specific phosphorylation sites Ser-795, Ser-780, and Ser-807/811, from New England Biolabs.

Immune Complex Kinase Assay.—Cells were suspended in lysis buffer containing 50 mM HEPES, pH 7.5, 200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 1 mM DTT, 0.1% Tween 20, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 μ g of leupeptin per ml, 5 μ g of aprotinin per ml, 10 mM β -glycerophosphate, 1 mM sodium orthovanadate, and 50 mM NaF, followed by a 10-s sonication and clearing by centrifugation at 14,000 rpm for 15 min. Supernatants were assayed for protein concentration by the Bradford assay (Bio-Rad); protein samples of 4 mg each were then immunoprecipitated for at least 2–4 h at 4 $^{\circ}$ C with protein A-agarose beads pre-coated with saturating amounts of the appropriate antibody (5 μ g). Immunoprecipitated proteins on beads were washed three times with 1 ml of lysis buffer and twice with kinase buffer (50 mM HEPES, pH 7.5, 1 mM DTT, 10 mM MgCl₂, plus protease inhibitors, as described above). The beads were resuspended in 50 μ l of kinase buffer containing 2 μ g of glutathione S-transferase (GST)-Rb-(769–921) fusion protein (Santa Cruz Biotechnology, Inc), 2.5 mM EGTA, 10 mM β -glycerophosphate, 1 mM Na₂VO₄, 20 μ M ATP, and 10 μ l of [³²P]ATP (8000 Ci/mmol, ICN). After incubation for 30 min at 30 $^{\circ}$ C, the samples were boiled in 2 $\times$ Laemmli buffer and separated by SDS-polyacrylamide gel electrophoresis. Phosphorylated proteins were visualized by exposure to phosphorstorage screens. The antibodies used were as follows: for cyclin D3 and cdk4, polyclonal rabbit antibodies were obtained from Santa Cruz Biotechnology.

RESULTS

The subcellular distribution of the two subtypes of the PLC β_1 , a and b, was evaluated by Western blot analysis in wild type erythroleukemic Friend cells (Fig. 1B). Although the PLC β_1 b is almost entirely localized in the nucleus, the PLC β_1 a is nearly equally distributed among the two compartments. The Western blot analysis of the clones overexpressing the PLC β_1 a, PLC β_1 b, and the mutant for the nuclear localization sequence M2b, respectively, show that whereas the M2b mutant has lost the capacity for nuclear localization, the clones overexpressing the two subtypes β_1 a and β_1 b have the PLC in the nucleus, the b form being entirely nuclear and the a form distributed in both nucleus and cytoplasm, even though in the latter compartment it is less expressed (Fig. 1A). The expression of PLC β_1 forms in wild type cells is very low since the immunoprecipitated PLC from 500 μ g of both nuclear and

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cytoplasmic protein gave rise to a signal even lower than the one obtained with 10 μ g of protein from nuclei or cytoplasm of transfected cells. The PLC activity of the clones overexpressing PLC β_1 a and M2b mutant as well as the same reported in Ref. 9 (i.e. PLC β_1 a 38.0 $\pm$ 1.5, M2b 9.3 $\pm$ 0.7 nmol of IP $_2$ /mg of protein/30 min of incubation). These values compared with nuclear PLC activity in wild type cells (9.8 $\pm$ 0.6 nmol of IP $_2$ /mg of protein/30 min of incubation) show a nearly 3-fold increase of the nuclear activity in the case of PLC β_1 a and PLC β_1 b (32.7 $\pm$ 2.0 nmol of IP $_2$ /mg of protein/30 min of incubation) with no changes in the case of PLC M2b, which conversely is 3-fold higher in the cytoplasm.

These features characterize all the clones we have selected for each type of transfectant and show a complete agreement between the enzymatic activity and the level of expression of the PLC β_1 forms. To determine whether PLC β_1 is involved in cell cycle progression, we examined cyclin D3, cyclin E, cdk2, and cdk4 protein expression in serum-starved cells and after stimulation with serum. In erythroleukemic Friend cells cultured without inducer, the protein levels of cyclin D3 and E fluctuate during the cell cycle, whereas the level of cyclin D2

changes little. Cyclin D1 protein is not detected in these cells. Cyclin D3 increases as cells progress through G $_1$ phase and reaches a maximum level in S phase. Cyclin E peaks at the transition from G $_1$ to S phase (21). Fig. 2 shows that in nuclear extracts of Friend cells, cyclin D3 with its related cdk4 and cyclin E were expressed at barely detectable levels in serum-starved wild type cells and accumulated after stimulation with serum, whereas the level of cdk2 protein, whose activity is low in G $_1$ phase and increases during S phase (21), is constant after serum stimulation. On the contrary, the clones overexpressing in the nucleus both PLC β_1 a and PLC β_1 b show high levels of cyclin D3 and cdk4 in serum-starved cells, whereas M2b mutant clone and wild type cells as well do not. Since the expression of cyclin E does not increase in serum-starved transfectants overexpressing PLC β_1 a or PLC β_1 b, we thought that overexpression of enzymatically active forms of PLC β_1 in the nucleus could exert a specific effect, via cyclin D3-cdk4 complex formation, in sustaining cell cycle progression even in the absence of serum. Therefore, we have evaluated the activity of the cyclin D3-cdk4 complex *in vitro* in order to establish whether the complex is enzymatically active. Cyclin D3 and cdk4 were immunoprecipitated from total cell lysates, and immune complex was then used for *in vitro* kinase assay by using a bacterially produced glutathione S-transferase-Rb fusion protein as a substrate. Fig. 2C shows that the activity of cyclin D3-cdk4 complex, which is almost absent in serum-starved wild type cells and M2b clone, increases after serum stimulation. On the contrary, in the clones overexpressing PLC β_1 a and -b in the nucleus the activity of the complex is present even after serum starvation, and its level is similar to that of both wild type cells and M2b clone after serum stimulation. This *in vitro* feature prompted us to analyze the *in vivo* phosphorylation status of pRb. We have evaluated pRb phosphorylation by using specific antibodies that detect specific phosphorylated sites as follows: Ser-780, Ser-795, and Ser-507/811 (New England Biolabs). When fresh medium with 10% serum was added for 12 h to serum-starved Friend cells (wild type cells), a significant increase of pRb Ser phosphorylation was observed on Ser-795, Ser-780, and Ser-807/811 as physiologically occurs (Fig. 3). Surprisingly transfectants overexpressing both forms of PLC β_1 in the nucleus show high levels of pRb phosphorylation, only on

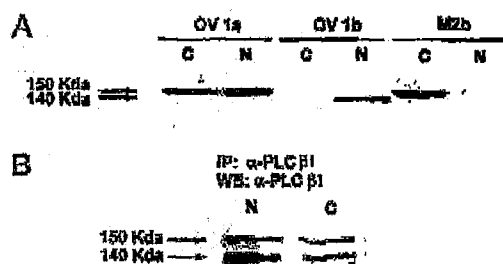
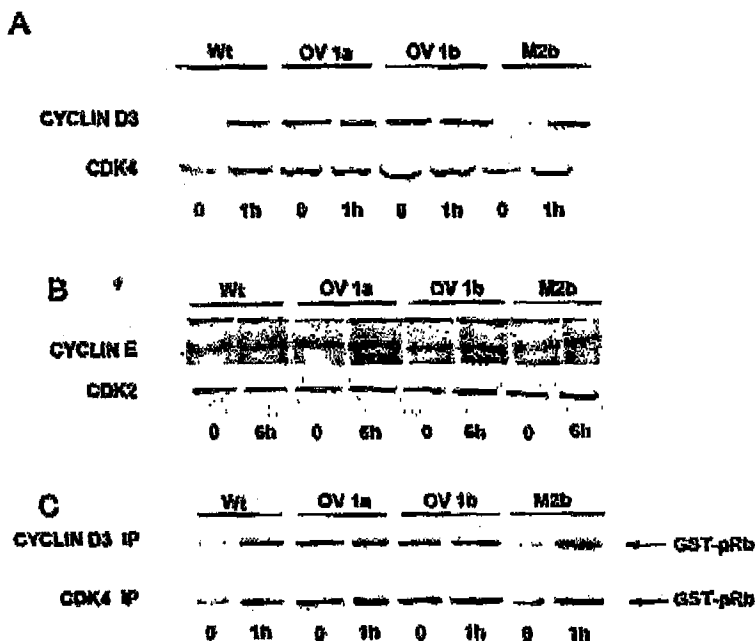


FIG. 1. Analysis of PLC β_1 phenotype in stable transfectants and Friend cells wt. A, immunoblot analysis of 10 μ g of nuclear and cytoplasmic lysate of the stable transfectants as follows: OV1a, cells transfected with PLC β_1 a; OV1b, cells transfected with PLC β_1 b; M2b, cells transfected with the mutant lacking the nuclear localization sequence. B, immunoblot analysis of 500 μ g of nuclear (N) and cytoplasmic (C) fractions of wt immunoprecipitated (IP) with 2.5 μ g of anti-PLC β_1 (a and b) (Signal Transduction). The Western blots (WB) and PLC activities of stable transfectants reported here are representative of 5 other clones, which behave exactly as the ones in the figure.

FIG. 2. Effect of overexpression of PLC β_1 subtypes (a and b) and the mutant M2b on both the expression and the activity of cell cycle proteins. A, changes in the level of expression of cyclin D3 and cdk4 in the presence or absence of serum. B, changes in the level of expression of cyclin E and cdk2 in the presence or absence of serum. C, changes in the activity of cyclin D3-cdk4 complex in the presence or absence of serum. Cells transfected with PLC β_1 a (OV1a), cells transfected with PLC β_1 b (OV1b), cells transfected with the mutant lacking the nuclear localization sequence (M2b), and wild type cells (Wt) were serum-deprived for 48 h and then stimulated with fresh serum (10%) for the indicated time. The Western blots and the activity assay of stable transfectants reported here are representative of 5 other clones, which behaved in an identical fashion.



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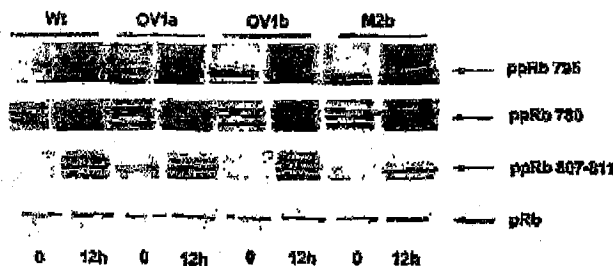
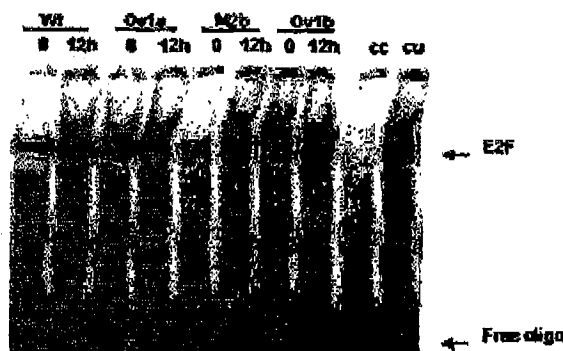


Fig. 2. Effect of overexpression of PLCβ₁ subtypes and the mutant M2b on pRb phosphorylation. Serum-starved cells, as described in the legend to Fig. 2, were diluted at 2 × 10⁶ with fresh medium containing 10% fetal calf serum for 12 h after which time pRb phosphorylation was found to peak. Abbreviations for wild type cells and clones are as in the previous figures. The membrane was probed with anti-pRb (New England Biolabs) and with anti-ppRb for specific phosphorylation sites, i.e. Ser-795, Ser-780, and Ser-807/811 (New England Biolabs) in a 7% polyacrylamide gel. The phosphorylated form of Rb protein is indicated by the designation ppRb, the form not phosphorylated is indicated by pRb. The Western blots of stable transfectants reported here are representative of 5 other clones, which behaved in an identical fashion.

Ser-795, even during serum starvation, whereas this does not occur at all in both the M2b mutant and the wild type cells (Fig. 3). Indeed cells overexpressing PLCβ₁ in the nucleus show phosphorylated pRb (Ser-795) after serum starvation for 48 h. The two antibodies against phosphorylation sites Ser-780 and Ser-807/811 did not reveal any phosphorylation in serum-starved clones overexpressing PLCβ₁ in the nucleus. The phosphorylation in starved clones, overexpressing PLCβ₁ in the nucleus, is restricted to Ser-795 possibly indicating a specific effect of the nuclear localization of PLCβ₁. It is concluded that the overexpression of PLCβ_{1a} and -b in the nucleus up-regulates both the expression and the activity of the cyclin D3-cdk4 complex and affects pRb phosphorylation. The absence of these two events in cells overexpressing the same PLC in the cytoplasmic compartment indicates a very specific effect linked to the subcellular localization of this signaling PLC.

The Rb proteins bind to a number of cellular proteins like E2F that are involved in the regulation of transcription of genes relevant for the control of the cell cycle. E2F transcription factors regulate the transition from the G₁ to S phase, and its activity is regulated by members of pRb family (22) and appears to be a downstream step of the signaling cascade that takes place in G₁ phase. To assess whether overexpression of the two subtypes of PLCβ₁ could affect functional changes in E2F activity, we evaluated E2F DNA binding activity in resting cells and in cells after stimulation with serum. Fig. 4 shows the effect of the overexpression of PLCβ_{1a} and -b on E2F-1 binding (19). As expected, in serum-starved clones overexpressing the two nuclear subtypes of PLCβ₁ the binding activity was higher than in wild type cells and M2b mutant cells, suggesting that PLCβ_{1a} and -b when localized in the nucleus are indeed involved in the regulation of proteins responsible for the progression of cell cycle. To address whether, after serum starvation, pRb is bound to E2F-1 in both wild type cells and M2b clone and not in serum-starved cells expressing PLCβ_{1a} and -b in the nucleus, we have added anti-pRb antibody in the binding assay. Fig. 4 shows a change in the mobility of the E2F-1 complex only in serum-starved wild type cells and M2b mutant clone. These observations indicate that the E2F-1 transcription factor is already released from pRb in serum-starved Friend cells overexpressing PLCβ_{1a} or -b in the nucleus. Given that E2F transcription factors play an integral role in cell cycle progression by inducing the expression of genes required for S phase entry, we checked the effect of the overexpression of PLCβ_{1a}

A



B

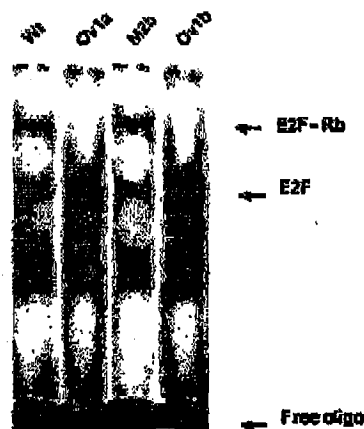


Fig. 4. Effect of PLCβ_{1a}, PLCβ_{1b}, and PLCβ₁M2b overexpression on E2F-DNA binding activity. Abbreviations for wild type cells and clones are as in the previous figures. Serum-starved cells (48 h) were stimulated by addition of fresh serum for 12 h. E2F-DNA complex formation was determined by DNA gel mobility shift assay (A). For antibody perturbation supershift experiments, anti-pRb antibody was added to the incubation in samples from serum-starved wild type cells and clones expressing PLCβ₁ (B). cc, preincubation with unlabeled E2F-1 oligonucleotide; cu, preincubation with nonspecific competitor. The stable transfectants reported here are representative of 5 other clones, which behaved in an identical fashion.

and -b and M2b mutant on the behavior of the cell cycle in these transfectants by means of flow cytometry. In the presence of serum the clones overexpressing PLCβ_{1a} and -b show a cell cycle profile characterized by a higher S phase compared with wt and the M2b mutant (Fig. 5). Moreover, when the clones overexpressing PLCβ_{1a} and -b in the nucleus are serum-starved, they still maintain a higher S phase compared with wt and M2b cells, the flow cytometric analysis of which shows an almost complete block in G₁ phase. Therefore, cells overexpressing the two subtypes of PLCβ₁ could at least in part effectively replace the requirement for serum as a mitogenic signal.

DISCUSSION

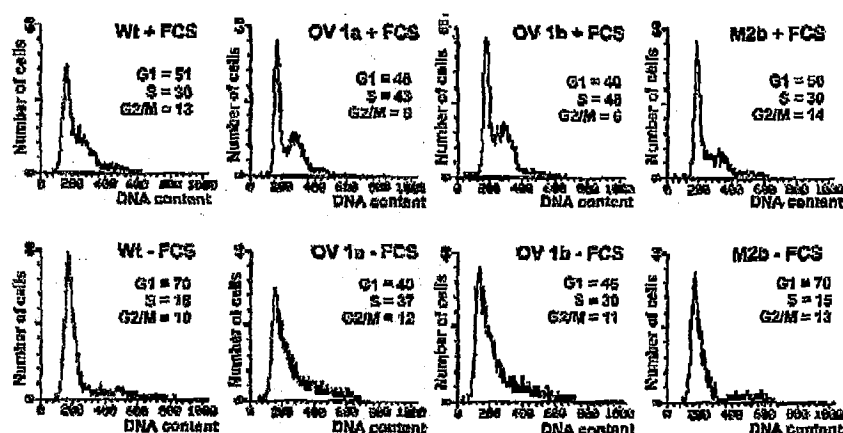
Nuclear PLCβ₁ signaling was suggested to be one of the earliest events following both exposure of human osteosarcoma Saos cells to interleukin-1α (23) and murine 3T3 cells to IGF-1 (18). A decrease in nuclear PIP₂ hydrolysis and down-regulation of nuclear PLCβ₁ have been detected during differentiation of erythroleukemia cells (24). Moreover, we have shown that nuclear PLCβ₁ is involved in maintaining the undifferen-

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Nuclear PLC β_1 and Cell Cycle

FIG. 5. Enforced PLC β_1 expression is sufficient to promote cell cycle progression. Cells were grown with 10% fetal calf serum (FCS) for 48 h and then starved for 24 h. Abbreviations for wild type cells and clones are as in the previous figures. Both in the presence and absence of serum, clones overexpressing nuclear PLC β_1 show a higher S phase as compared with wild type and M2b-transfected clones. The stable transfectants reported here are representative of 5 other clones, which behaved in an identical fashion.



tiated state of Friend erythroleukemia cells, possibly by opposing the inhibition of the cell cycle progression necessary for erythroid differentiation (9). Thus this isoform is a good candidate as a key element for the signaling cascade involved in cell growth and proliferation. The contention that subcellular localization of the inositol lipid cycle is central to the function of the cycle itself in controlling the signaling events has been strengthened by the discovery that in *Saccharomyces cerevisiae* a nuclear PLC, called PLC1, homologous in function to the mammalian PLC β_1 , and two inositol polyphosphate kinases constitute a signaling pathway that affects transcriptional control (25). Our study was intended to find out how nuclear PLC β_1 is involved in the regulation of cellular proliferation. To address this issue we have tried to identify the targets of nuclear PLC β_1 in the nuclear compartment. Therefore we first examined the expression of cyclins and cdk4 associated with G₁-S phase transition. Our evidence suggests that the overexpression of PLC β_1 in the nucleus is directly responsible for the overexpression and activation of cyclin D3-cdk4 complex, which is known to stimulate progression through G₁ rather than to promote the G₁-S transition (11). This is indeed a specific target, given that the other cyclin, cyclin E, which is responsible for G₁-S transition (26) is not affected at all by nuclear PLC β_1 . The two splicing products of this isoform behave in the same fashion, even though the 1b is more specific for nuclear localization and 1a localizes also in the cytoplasmic compartment. This suggests that once PLC β_1 is in the nucleus its role is different from its counterpart located at the plasma membrane. This is supported by the absence of an effect following overexpression of the M2b mutant in which PLC localizes exclusively to the cytosolic compartment (8, 9). In this case the effects on both cyclin D3 and cdk4 as well are totally absent. Only the clones overexpressing both PLC β_1 a and -b, but not the M2b mutant, show a higher S phase compared with wild type and M2b-transfected clones both in cycling and serum-starved cells. This implies that, since cyclin E is not affected, both the overexpression and the activation of the cyclin D3-cdk4 complex are actually responsible for maintaining the cell cycle progression. Downstream events such as the phosphorylation of pRb and the subsequent release of E2F-1 transcription factor are also only observed in PLC β_1 a and -b clones after serum starvation.

It should be noted that the overexpression of PLC β_1 in the nucleus gives rise to enzymatically functional PLC whose activity is 3-fold higher than the one of wt cells. This enhanced signaling capacity generates second messengers such as diacylglycerol, whose role in nuclear protein kinase C activation has been widely explored (19, 27, 28) and inositol trisphosphate,

whose nuclear kinases, by producing the other inositol polyphosphates, are involved in mRNA export and transcription in yeast (25). Thus, data reported here appear to shed new light on the lipid-mediated signaling machinery residing in the nucleus, implicating nuclear PLC β_1 as a key element for the specific regulation of the cyclin D3-cdk4 complex and its downstream targets such as pRb and E2F in facilitating cell cycle progression.

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Total Synthesis of Caloporoside

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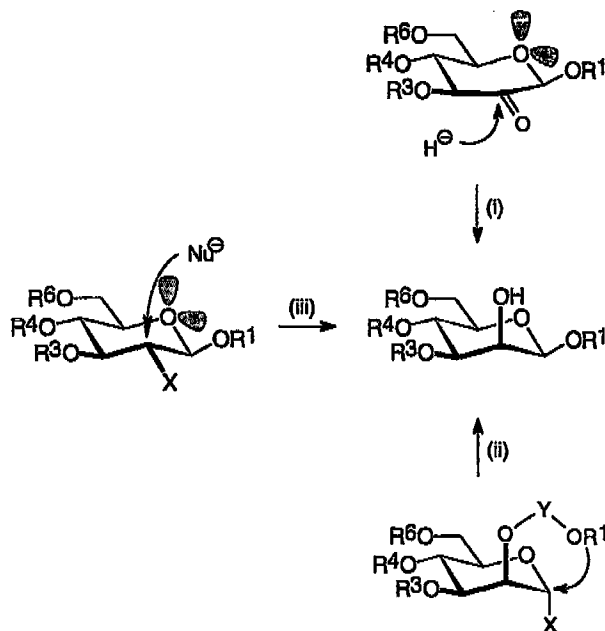
Received January 5, 1998

The first total synthesis of the fungal metabolite caloporoside **1**, a strong and selective inhibitor of phospholipase C, is described. Both sugar units of its complex disaccharidic segment were obtained from 3,4,6-tri-*O*-benzyl-D-glucopyranose **14** as a common building block, with D-glucose → D-mannose inversions as the key strategic elements. This particular substitution reaction occurred readily on the acyclic segment (**27** → **28**), whereas ultrasonication was required to override adverse stereoelectronic effects upon formation of β-D-mannopyranoside unit **34**. The (16*R*)-hydroxyheptadecylsalicylic acid part of **1** was efficiently prepared by a palladium-catalyzed Suzuki cross coupling reaction of aryltriflate **7** with the 9-alkyl-9-BBN derivative formed from alkene **6** and 9-H-9-BBN.

Introduction

The efficient formation of β-D-mannopyranosidic linkages constitutes one of the ultimate challenges in oligosaccharide synthesis.¹ Conventional glycosylation strategies exploiting the anomeric effect of a pyranoside template and/or the anchimeric assistance of proper substituents on O-2 strongly disfavors this particular type of 1,2-cis arrangement. Despite some progress in direct β-D-mannoside syntheses² as well as in enzymatic mannosylation reactions,³ the most efficient entries into this particular type of glycoside still rely on indirect methods (Scheme 1). Notable among them are (i) stereoselective reductions of β-D-arabino-hexopyranos-2-ulosides formed either directly via ulosyl bromide donors⁴ or indirectly by oxidation of 2-O-unprotected β-glucosides,⁵ (ii) well-designed molecular arrays using temporary linkers "Y" which transfer the aglycon in an entropically favored intramolecular process to the β-side of the glycosyl donor ("intramolecular aglycon delivery"),⁶ and (iii) β-D-glucose → β-D-mannose inversions via S_N2 processes.^{7,8}

Scheme 1



While strategies (i) and (ii) usually require protecting group manipulations prior to the glycosylation event and are restricted to fairly reactive glycosyl acceptors R¹OH, method (iii) suffers from adverse stereoelectronic effects

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and each phenyl moiety of benzyl and benzhydryl may be substituted by C₁₋₆alkyl, C₁₋₆alkoxy, halo or trifluoromethyl; and

5 R³ represents H, COR⁹, CO₂R¹⁰, COCONR¹⁰R¹¹, COCO₂R¹⁰, SO₂R¹⁵, CONR¹⁰SO₂R¹⁵, C₁₋₆alkyl optionally substituted by a group selected from (CO₂R¹⁰, CONR¹⁰R¹¹, hydroxy, cyano, COR⁹, NR¹⁰R¹¹, C(NO₂)NR¹⁰R¹¹, CONHphenyl(C₁₋₄alkyl), COCO₂R¹⁰, COCONR¹⁰R¹¹, SO₂R¹⁵, CONR¹⁰SO₂R¹⁵ and phenyl optionally substituted by one or
10 more substituents selected from C₁₋₆alkyl, C₁₋₆alkoxy, halo and trifluoromethyl), Y-R⁸ or CO-Z-(CH₂)_q-R¹²;

R⁴, R⁵, R⁶ and R⁷ each independently represent H or C₁₋₆alkyl;

15 R⁸ represents an optionally substituted aromatic heterocycle;

R⁹ represents H, C₁₋₆alkyl or phenyl;

R¹⁰ and R¹¹ each independently represent H or C₁₋₆alkyl;

20 R¹² represents NR¹³R¹⁴ or an optionally substituted aromatic or non-aromatic azacyclic or azabicyclic group;

R¹³ and R¹⁴ each independently represent H, C₁₋₆alkyl, phenyl optionally substituted by one or more of C₁₋₆alkyl, C₁₋₆alkoxy, halo or trifluoromethyl or
25 phenylC₁₋₄alkyl optionally substituted in the phenyl ring by one or more of C₁₋₆alkyl, C₁₋₆alkoxy, halo or trifluoromethyl;

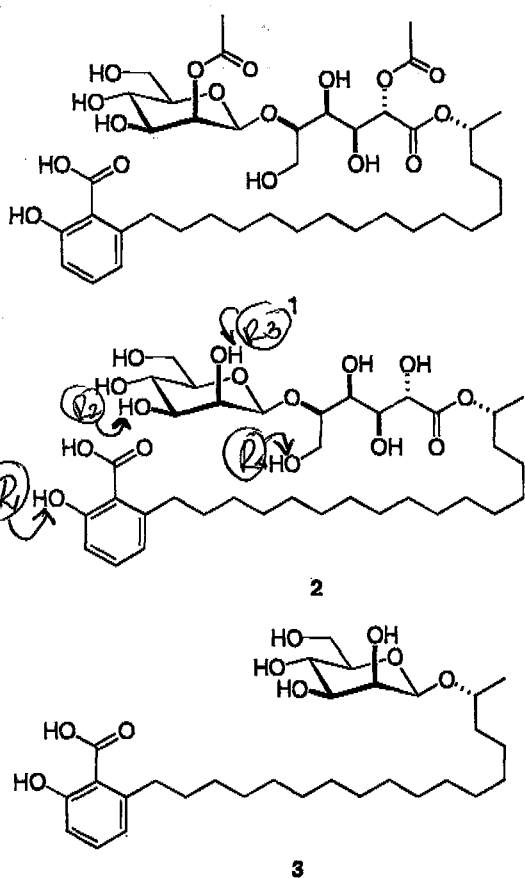
30 R¹⁵ represents C₁₋₆alkyl, trifluoromethyl or phenyl optionally substituted by one or more substituents selected from C₁₋₆alkyl, C₁₋₆alkoxy, halo and trifluoromethyl;

Y represents a hydrocarbon chain of 1,2,3 or 4 carbon atoms which may optionally be substituted by oxo;

Z represents CH₂, O, S or NR¹⁰; and

of the ring oxygen atom along the trajectory of the entering nucleophile, which generally impede nucleophilic substitutions at C-2.

With this background in mind, the synthesis of caloporoside 1,⁹ a secondary metabolite isolated from *Caloporous dichrous*, represents a formidable task. The major problem is obviously posed by its β -D-mannopyranosyl-D-mannonic acid part, although further complications arise from the acylation pattern of this saccharidic segment: Only the axially oriented (and hence rather unreactive) O-2' of the mannoside as well as the O-2 position of the acyclic mannonate unit are acetylated. Therefore the attentive choice of orthogonal protecting groups throughout the synthesis is of eminent importance for the regioselective introduction of the OAc residues as well as for avoiding any migration or cleavage of these esters during the final deprotection steps.

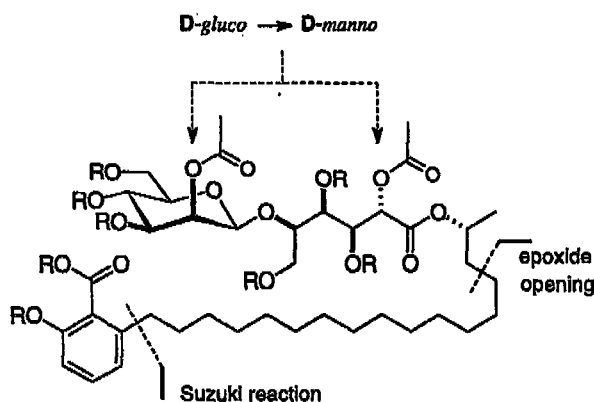


Caloporoside itself, as well as its deacylated analogue 2 and the monosaccharidic congener 3, which were both isolated from a culture broth of the fungus HA 137-89,¹⁰ are very attractive targets for total synthesis due to their pronounced physiological effects. Specifically, 1 has been shown to be a strong and selective inhibitor of phospholipase C, whereas 2 and 3 are inhibitors for the GABA_A/benzodiazepine chloride channel receptor complex and thus constitute neurotransmitter modulators of potential pharmacological relevance.

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Scheme 2



Tatsuta et al. have recently reported syntheses of 2 and 3.¹¹ In parallel work, we have outlined a modular entry into their common 6-alkylsalicylic acid "aglycon" as well as an efficient synthesis of compound 3 as the elementary member of this series of fungal metabolites based on the "ulosyl bromide strategy" (Scheme 1, method i) for the construction of the β -D-mannosidic linkage.¹² We now disclose the first total synthesis of caloporoside 1 itself with the proper acylation pattern, which relies on a highly efficient β -D-glucose $\rightarrow$ β -D-manno inversion promoted by ultrasound as the key step.

Results and Discussion

Retrosynthetic Analysis and Formation of the Building Blocks. Our retrosynthetic analysis is summarized in Scheme 2. The formation of the salicylic acid part essentially follows the route outlined previously: thus, (*R*)-propenoxide 4 delivers the chiral information and a Suzuki cross coupling is used to attach the alkyl chain to the salicylic acid nucleus.¹² Furthermore, we envisage two D-glucose $\rightarrow$ D-manno inversions to afford the proper stereochemistry in the sugar portion of 1. This strategy has the inherent advantage that both units of the disaccharide can be traced back to the same glucose-derived precursor. By choosing benzyl protecting groups throughout this convergent synthesis, any scrambling of the acetyl residues of caloporoside in the final deprotection step should be precluded.

Reaction of 13-tetradecenylmagnesium bromide with (*R*)-propenoxide 4¹³ catalyzed by CuCl(COD) followed by silylation of the resulting enantiomerically pure alcohol 5 cleanly affords alkene 6 (Scheme 3). Its hydroboration with 9-H-9-BBN dimer and subsequent addition of NaOMe leads to a borate complex which undergoes an efficient cross-coupling reaction with aryl triflate 7 in the presence of PdCl₂(dppf) (2.5 mol %) and KBr as a stabilizing agent.^{12,14} Treatment of 8 with the sodium salt of benzyl alcohol readily cleaves the isopropylidene group and affords the corresponding benzyl ester which is protected at the phenolic OH with BnBr/K₂CO₃ to provide compound 9. Desilylation of the latter leads to alcohol 10 in excellent yield which is used as a first key building block for the assembly of 1.

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(13) Now readily available according to Tokunaga, M.; Larrow, J. F.; Kakiuchi, F.; Jacobsen, E. N. *Science* **1997**, *277*, 936.

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4-(4-methoxyphenyl)-4-[(3,5-bis(trifluoromethyl)benzyloxymethyl)piperidine hydrochloride;

4-phenyl-4-[(2-methoxy-5-bromo benzyloxymethyl)piperidine hydrochloride;

4-phenyl-4-[1-(3,6-dichlorophenyl)-ethoxymethyl]piperidine hydrochloride;

4-phenyl-4-[1-(2,3-dichlorophenyl)-ethoxymethyl]piperidine hydrochloride;

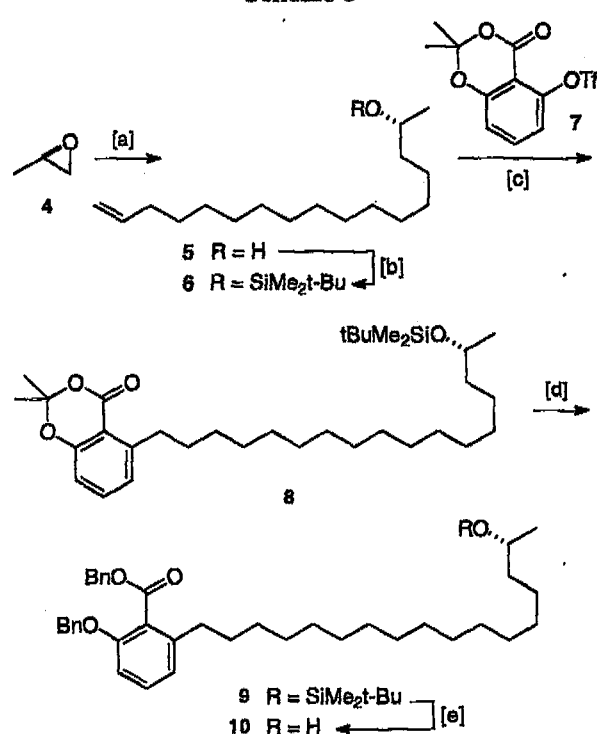
4-phenyl-4-[2,3-(dimethoxy)benzyloxymethyl]piperidine hydrochloride;
and salts thereof.

10. The use of a compound of the formula (I) as defined in any of claims 1 to 9 or a pharmaceutically acceptable salt or a prodrug thereof in therapy.

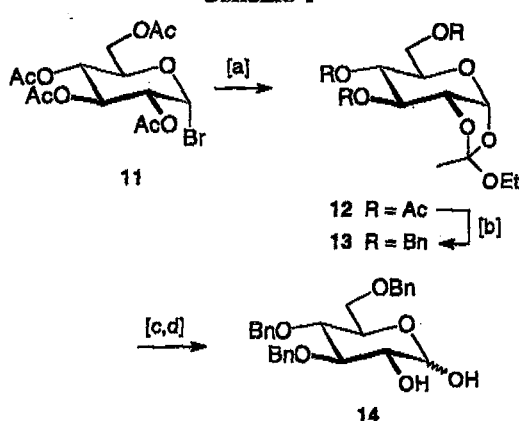
11. The use of a compound of formula (I) as defined in any of claims 1 to 9 or a pharmaceutically acceptable salt thereof for use in the manufacture of a medicament for the treatment of physiological disorders associated with any excess of tachykinins, especially substance P.

12. ~~A pharmaceutical composition comprising a compound of the formula (I) as defined in any of claims 1 to 9 or a pharmaceutically acceptable salt thereof in association with a carrier thereof.~~

13. A method for the treatment or prevention of physiological disorders associated with an excess of tachykinins, especially substance P, which method comprises the administration to a patient in need thereof a tachykinin reducing amount of a compound of formula (I)

Scheme 3^a

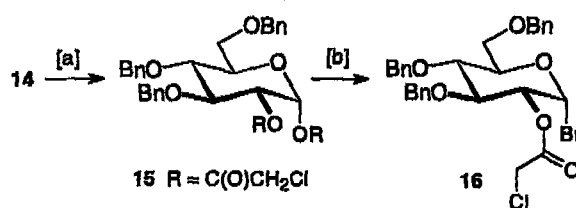
^a [a] 13-tetradecenylmagnesium bromide, CuCl(COD) (10 mol %), THF, -78 °C → rt, overnight, 86%; [b] TBDMS-OTf, imidazole, DMF, rt, 94%; [c] (i) [9-H-9-BBN]₂ (0.5 equiv), THF; (ii) NaOMe (1 equiv), KBr (1.1 equiv), PdCl₂(dppf) (2.5 mol %), THF, reflux, overnight, 86%; [d] (i) BnOH, NaH, DMF, 3 h, rt, 92%; (ii) BnBr, K₂CO₃, DMF, 4 h, reflux, 93%; [e] TBAF, THF, rt, 89%.

Scheme 4^a

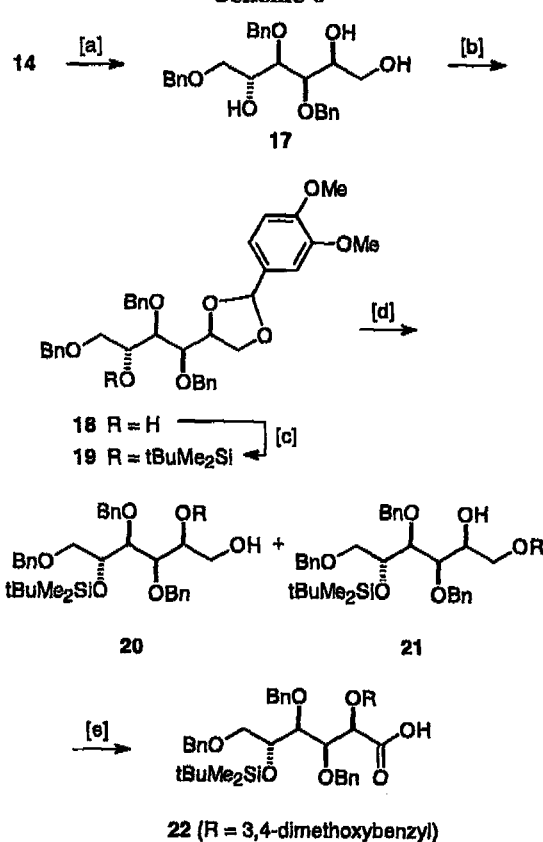
^a [a] Bu₄NBH₄, *s*-collidine, EtOH, 85%; [b] BnBr, KOH, THF, 81%; [c] 2 N HCl, THF; [d] K₂CO₃, MeOH, 1 h, rt, 87% over two steps.

Acetobromoglucose 11 is converted into 3,4,6-tri-O-benzyl-D-glucopyranose 14 in four conventional steps on a multigram scale by closely following a literature protocol.^{4a} This compound serves as a common intermediate for both sugar units of caloporoside (Scheme 4).

The second building block was obtained from 14 by simple acylation with chloroacetic acid anhydride and subsequent displacement of the anomeric ester group with HBr/HOAc. The crude glycosyl bromide 16 thus obtained was used without further purification (Scheme 5).

Scheme 5^a

^a [a] (ClCH₂CO)₂O, pyridine, -20 → -10 °C, 1.5 h, 85%; [b] HBr/HOAc, CHCl₃, 0 °C → rt, 3 h, 95%.

Scheme 6^a

^a [a] NaBH₄, THF/H₂O, 0 °C → rt, 2 h, 96%; [b] 3,4-dimethoxybenzaldehyde, H₂SO₄ cat., MS 3 Å, CH₂Cl₂, 3.5 h, 84%; [c] tBuMe₂SiCl, imidazole, DMF, 15 h, rt, 91%; [d] BH₃·THF, THF, reflux, 2 h, 20 (62%) + 21 (32%); [e] (i) Swern oxidation; (ii) NaClO₂, HSO₃NH₂, THF/H₂O, rt, 10 min, 93%.

Reduction of 14 with NaBH₄ provides triol 17 which is transformed into the dimethoxybenzylidene acetal 18 and then silylated as shown in Scheme 6. Subsequent reductive cleavage of the acetal in 19 is best achieved with BH₃·THF,¹⁵ affording the desired primary alcohol 20 in 62% yield together with 32% of the regioisomeric product 21; the latter can be readily separated by flash chromatography. Reducing agents other than borane for the cleavage of the acetal did not improve the isomeric

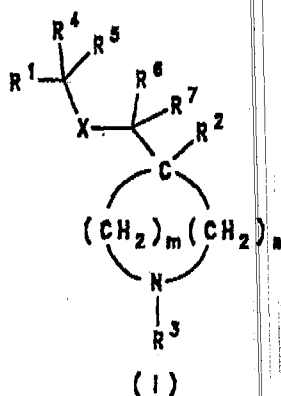
(14) Review: (a) Miyaura, N.; Suzuki, A. *Chem. Rev.* 1995, 95, 2457. For applications of Suzuki reactions from our laboratory, see: (b) Fürstner, A.; Seidel, G. *Tetrahedron* 1995, 51, 11165. (c) Fürstner, A.; Seidel, G.; Gabor, B.; Kopske, C.; Krüger, C.; Mynott, R. *Tetrahedron* 1995, 51, 8875. (d) Fürstner, A.; Nikolakis, K. *Liebigs Ann.* 1996, 2107.

(15) (a) Fleming, B.; Bolker, H. I. *Can. J. Chem.* 1974, 52, 888. (b) See also: Tsuru, T.; Kamata, S. *Tetrahedron Lett.* 1985, 26, 5195.

- 3 -

In essence, this invention provides a class of potent non-peptide tachykinin antagonists.

The present invention provides a compound of formula (I), or a salt or prodrug thereof:



wherein

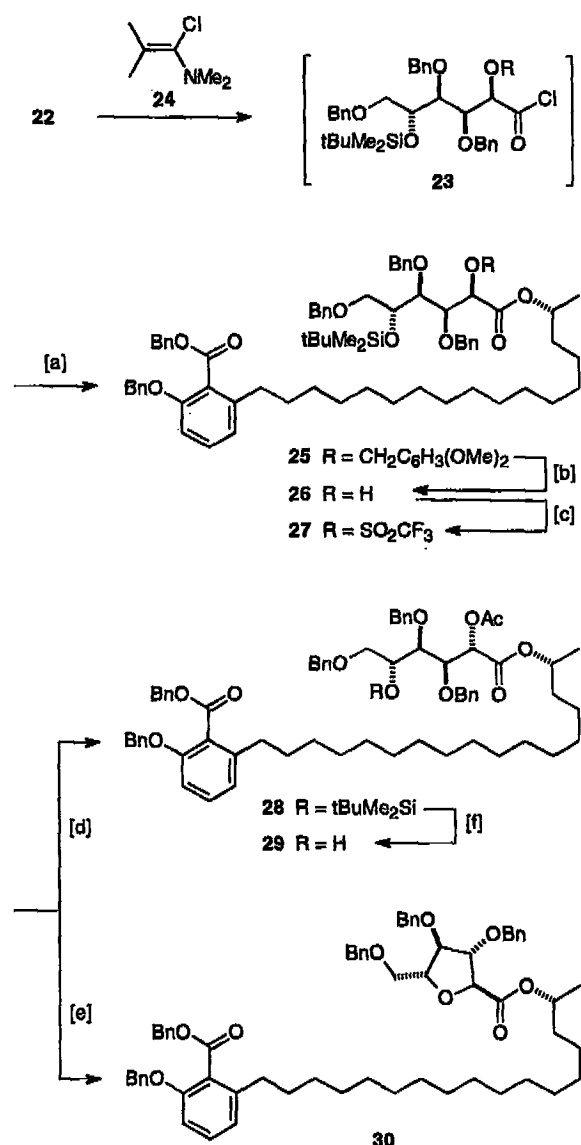
- 15 m is 2, 3 or 4;
 n is 0, 1 or 2 when m is 2 or 3, and n is 0 or
 1 when m is 4;
 X represents O or S;
 R¹ represents phenyl optionally substituted by
 20 1, 2 or 3 groups selected from C₁-6alkyl, C₂-6 alkenyl,
 C₂-6alkynyl, halo, cyano, nitro, trifluoromethyl,
 trimethylsilyl, -OR^a, SR^a, SOR^a, SO₂R^a, -NR^aR^b, -NR^aCOR^b,
 -NR^aCO₂R^b, -CO₂R^a or -CONR^aR^b, where R^a and R^b each
 independently represent H, C₁-6alkyl, phenyl or
 25 trifluoromethyl;
 R² represents phenyl optionally substituted by
 1, 2 or 3 groups selected from C₁-6alkyl, C₂-6 alkenyl,
 C₂-6alkynyl, halo, cyano, nitro, trifluoromethyl,
 trimethylsilyl, -OR^a, SR^a, SOR^a, SO₂R^a, -NR^aR^b, -NR^aCOR^b,
 30 -NR^aCO₂R^b, -CO₂R^a or -CONR^aR^b, where R^a and R^b are as
 previously defined; heteroaryl selected from indazolyl,
 thienyl, furyl, pyridyl, thiazolyl, tetrazolyl and
 quinolyl; benzhydryl; or benzyl; wherein each heteroaryl
 and each phenyl moiety of benzyl and benzhydryl may be

ratio but rather compromised the yields. Swern oxidation¹⁶ of **20** followed by exposure of the resulting crude aldehyde to NaClO₂/HSO₃NH₂¹⁷ in aqueous THF provides the properly protected gluconic acid **22** as the third building block for the synthesis of **1** (Scheme 6).

Assembly. With building blocks **10**, **16**, and **22** in hand, we tackled the assembly of the caloporoside skeleton. Thus, by using the chloroenamine reagent **24** developed by Ghosez et al.,¹⁸ it was possible to convert the gluconic acid **22** under *strictly neutral* conditions into the corresponding acid chloride **23**, which was immediately trapped with alcohol **10**. As it proved difficult to separate the resulting ester **25** from traces of unreacted starting materials by flash chromatography, crude **25** was deprotected by oxidative cleavage of the dimethoxybenzyl group with DDQ in CH₂Cl₂.¹⁹ The purification of the corresponding alcohol is readily achieved, leading to pure **26** in 74% yield over three steps without any racemization of the C-2 stereogenic center. Moreover, it should be mentioned that the use of the 3,4-dimethoxybenzyl group was a key to success: while this group was readily split off by DDQ, the corresponding 4-monomethoxybenzyl ether could not be cleaved under these conditions (Scheme 7).

Treatment of alcohol **26** with trifluoromethanesulfonic acid anhydride in pyridine/CH₂Cl₂ affords the rather unstable triflate **27** which readily reacts to the desired D-mannonic acid ester **28** on addition of KOAc in DMF (Scheme 7). Surprisingly though, we noticed a pronounced effect of the temperature on this process: while the intermolecular substitution proceeds cleanly at ambient temperature, an intramolecular reaction prevails at 0 °C leading to the formation of the tetrahydrofuran derivative **30** via attack of the 5-OTBDMS ether rather than external KOAc onto the triflate.²⁰

The desilylation of mannonate **28** turned out to be somewhat delicate: both the use of TBAF and of HF/MeCN lead to the concomitant cleavage of the mannonic acid ester, while pyridinium *p*-toluenesulfonate is totally ineffective. The use of BF₃·Et₂O in CH₂Cl₂, however, cleanly affords the desired alcohol **29** in 83% yield, which is glycosylated under modified Koenigs-Knorr conditions²¹ with glucosyl bromide **16** using Ag⁺ on silica/alumina as the promotor (Scheme 8).²² Since the anchimeric assistance exerted by the chloroacetyl group directs the glycosylation to the β-side, disaccharide **31** is obtained in good yield and excellent selectivity; the chloroacetyl

Scheme 7^a

^a [a] **10**, DMAP, CH₂Cl₂, 0 °C → rt, 16 h; [b] DDQ, CH₂Cl₂/H₂O, 0 °C → rt, 3.5 h, 74% (over last three steps); [c] triflic anhydride, CH₂Cl₂/pyridine, 0 °C, 15 min; [d] KOAc, DMF, rt, 1 h, 75%; [e] KOAc, DMF, 0 °C → rt, **30** (75%) + **28** (18%); [f] BF₃·Et₂O, CH₂Cl₂, 0 °C → rt, 1.5 h, 83%.

residue can then be selectively cleaved off with thiourea²³ without affecting the resident 2-O-acetyl substituent on the mannonate entity (**31** → **32**).

This sets the stage for the crucial β-D-glucopyranoside → β-D-mannopyranoside inversion process: formation of triflate **33** under standard conditions followed by treatment with Bu₄NOAc in toluene cleanly provides the desired 2-O-acetyl-β-D-mannopyranosyl-D-mannonic acid ester **34** without any formation of the undesired gluco isomer interfering, provided that the reaction is carried out *with ultrasonication* (Scheme 8). In the NMR spectra, the observed coupling constants *J*_{H1',H2'} ≈ 1 Hz and

(22) Van Boeckel, C. A. A.; Beetz, T.; Kock-van Dalen, A. C.; van Bakkum, H. *Recl. Trav. Chim. Pays-Bas* **1987**, *106*, 596.

(23) For precedence, see i.a.: Naruto, M.; Ohno, K.; Naruse, N.; Takeuchi, H. *Tetrahedron Lett.* **1979**, 251.

(16) Review: Mancuso, A. J.; Swern, D. *Synthesis* **1981**, 165.

(17) (a) Lindgren, B. O.; Nilsson, T. *Acta Chem. Scand.* **1973**, *27*, 888. (b) Colombo, L.; Gennari, C.; Santandrea, M.; Narisano, E.; Scolastico, C. *J. Chem. Soc., Perkin Trans. 1* **1980**, 136.

(18) (a) Devos, A.; Remion, J.; Frisque-Heshain, A.-M.; Colens, A.; Ghosez, L. *J. Chem. Soc., Chem. Commun.* **1979**, 1180. (b) Haveaux, B.; Dekoker, A.; Rens, M.; Sidani, A. R.; Toye, J.; Ghosez, L. *Org. Synth.* **1980**, *59*, 26.

(19) For a review, see: Greene, T. W.; Wuts, P. G. M. *Protective Groups in Organic Synthesis*, 2nd ed.; Wiley: New York, 1991.

(20) For a review on carbohydrate-derived triflates, see: (a) Binkley, R. W.; Ambrose, M. G. *J. Carbohydr. Chem.* **1984**, *3*, 1. (b) See also: Stang, P. J.; Hanak, M.; Subramanian, R. L. *Synthesis* **1982**, 85. (c) Carbohydrate-derived triflates are prone to ring contraction via intramolecular substitution reactions, cf. Binkley, R. W. *J. Org. Chem.* **1992**, *57*, 2353. (d) Wheatley, J. R.; Bichard, C. J. F.; Mantell, S. J.; Son, J. C.; Hughes, D. J.; Fleet, G. W. J.; Brown, D. J. *Chem. Soc., Chem. Commun.* **1993**, 1065 and references cited.

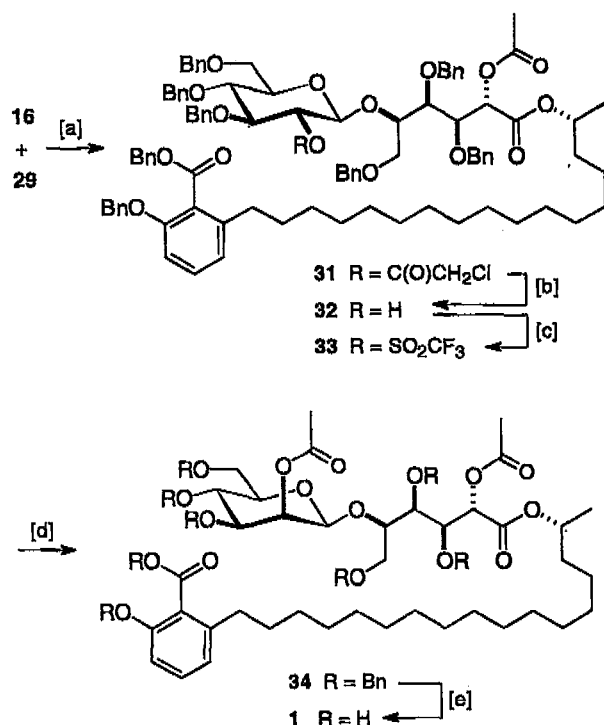
(21) For reviews, see: (a) Paulsen, H. *Angew. Chem.* **1982**, *94*, 184; *Angew. Chem., Int. Ed. Engl.* **1982**, *21*, 155. (b) Schmidt, R. R. In *Comprehensive Organic Synthesis*; Trost, B. M., Fleming, I., Eds.; Pergamon: Oxford, 1991; Vol. 6, p 33.

- 2 -

Tachykinin Receptor Antagonists", C.A. Maggi, R. Patacchini, P. Rovero and A. Giachetti, J. Auton. Pharmacol. (1993) 13, 23-93. Tachykinin antagonists are also believed to be useful in allergic conditions [Hamelet et al Can. J. Pharmacol. Physiol. (1988) 66 1361-7], immunoregulation [Lotz et al Science (1988) 241 1218-21 and Kimball et al, J. Immunol. (1988) 141 (10) 3564-9], and as anticonvulsants [Garant et al, Brain Research (1986) 382 372-8]. Tachykinin antagonists may also be useful in the treatment of small cell carcinomas, in particular small cell lung cancer (SCLC) [Langdon et al, Cancer Research (1992) 52, 4554-7].

It has furthermore been suggested that tachykinins have utility in the following disorders: depression, dysthymic disorders, chronic obstructive airways disease, hypersensitivity disorders such as poison ivy, vasospastic diseases such as angina and Reynauld's disease, fibrosing and collagen diseases such as scleroderma and eosinophillic fascioliasis, reflex sympathetic dystrophy such as shoulder/hand syndrome, addiction disorders such as alcoholism, stress related somatic disorders, neuropathy, neuralgia, disorders related to immune enhancement or suppression such as systemic lupus erythmatosis (European patent application no. 0 436 334), conjunctivitis, vernal conjunctivitis, contact dermatitis, atropic dermatitis, urticaria, and other eczematoid dermatitis (European patent application no. 0 394 989) and emesis (European patent application no. 0 533 280).

~~In view of their metabolic instability, peptide derivatives are likely to be of limited utility as therapeutic agents. It is for this reason that non-peptide tachykinin antagonists are sought.~~

Scheme 8^a

^a [a] Ag⁺ on silica/alumina, MS 3 Å, CH₂Cl₂, -5 °C, 45 min, 74%; [b] thiourea, Na₂CO₃, EtOH/CH₂Cl₂, reflux, 4 h, 75%; [c] triflic anhydride, pyridine, rt, 45 min, 78%; [d] Bu₄NOAc, toluene, ultrasound, 16 h, 95%; [e] H₂ (1 atm), Pd/C (5%), MeOH + HOAc (1%), 22 h, 96%.

$J_{C1',H1'}$ = 156.8 Hz confirm the β -D-*manno* arrangement.²⁴ Although similar ultrasound-assisted D-*gluco* $\rightarrow$ D-*manno* inversions using CsOAc have been recently described in the literature,^{7b,c} we would like to stress the fact that Bu₄NOAc exhibits a far superior reactivity in this difficult substitution reaction and ensures a much more efficient product formation. Furthermore, it should be noted that attempts to effect the inversion under thermal conditions either failed to afford any **34** or led to rather complex mixtures if the reactions were run under more forcing conditions.

Hydrogenolysis of the eight benzyl protecting groups in the fully protected mannoside **34** over Pd on charcoal in MeOH/HOAc (1%) affords caloporoside **1** in near quantitative yield and completes the first total synthesis of this demanding target. Its spectroscopic and analytical data are in full agreement with those reported in the literature.⁹

While previous synthetic studies were able to devise elegant and efficient routes to the deacetylated analogue **2**¹¹ and the monosaccharidic congener **3**^{11,12} of caloporoside, the synthesis outlined above is the first one to address the problems posed by the acylation pattern of this important target. The ultrasound-driven nucleophilic displacement of a 2-*O*-triflyl group by Bu₄NOAc installs the required β -D-mannosidic linkage and thereby solves the regiochemical issues. We are presently exploring the scope of this promising new approach to β -D-mannopyranosides in more detail.²⁵

(24) For the assignment of the anomeric configuration via $J_{C,H}$, see: Bock, K.; Pedersen, C. *J. Chem. Soc., Perkin Trans. 2* 1974, 293.

Experimental Section

General. All reactions were carried out under Ar in predried glassware using Schlenk techniques. The solvents were dried by distillation over the drying agents indicated and were stored and transferred under Ar: CH₂Cl₂ (P₄O₁₀), toluene (Na/K), THF (magnesium/anthracene), DMF (Desmodur®, Bayer AG; dibutyltin dilaurate), pyridine (KOH), EtOH (Mg), MeOH (Mg). Flash chromatography: Merck silica gel (230–400 mesh) using hexane/ethyl acetate in various proportions as eluent. For the instrumentation used and the spectra formats see ref. 25f. Mp: Gallenkamp apparatus (uncorrected). Elemental analyses: Dornis & Kolbe, Mülheim. Commercially available reagents (Aldrich, Fluka) were used as received.

2-Benzyloxy-6-[(16*R*)-*tert*-butyldimethylsilyloxy]-heptadecyl]benzoic Acid Benzyl Ester (9**).** Benzyl alcohol (10 mL) was added to a suspension of NaH (0.51 g, 22.18 mmol) in DMF (40 mL) at 0 °C, and the mixture was stirred for 30 min while being warmed to ambient temperature. Dioxinone **8** (4.00 g, 7.31 mmol)¹² was introduced and stirring was continued for 3 h. The suspension was neutralized with aqueous 1 N HCl, buffered with saturated aqueous NaHCO₃ (50 mL), and extracted with Et₂O (3 $\times$ 50 mL). The combined organic layers were washed with water (50 mL) and dried over Na₂SO₄, and the solvents were removed in vacuo. Flash chromatography (hexane/ethyl acetate 15:1 $\rightarrow$ 10:1) of the residue yielded 2-hydroxy-6-[(16*R*)-*tert*-butyldimethylsilyloxy]-heptadecyl]benzoic acid benzyl ester as a colorless oil (4.01 g, 92%): $[\alpha]_D^{20}$ -4.9° (c 8.1, CHCl₃); IR 3035, 2926, 2854, 1661, 1608, 1449, 1250, 1209, 835, 774; ¹H NMR (CDCl₃, 300 MHz) δ 7.34–7.45 (m, 5H), 7.25 (m, 1H), 6.82 (dd, J = 8.3, 1.2, 1H), 6.67 (dd, J = 7.5, 1.2, 1H), 5.36 (s, 2H), 3.75 (m, 1H), 2.79 (m, 2H), 1.10 (d, J = 6.1, 3H), 1.02–1.42 (br, 28H), 0.88 (s, 9H), 0.04 (s, 6H); ¹³C NMR (CDCl₃, 75 MHz) δ 171.3, 164.3, 162.8, 134.9, 134.2, 129.0, 128.7, 128.7, 122.4, 115.6, 111.8, 68.6, 67.6, 39.8, 36.8, 32.4, 29.8, 29.7, 29.6, 29.6, 25.9, 25.8, 23.8, 18.6, -4.4, -4.7; MS m/z (rel intensity) 539 ([M - C₄H₉]⁺, 7), 521 (32), 477 (5), 447 (5), 431 (46), 221 (9), 207 (9), 195 (7), 181 (3), 159 (9), 91 (100), 75 (12); C₃₇H₆₀O₅Si (596.96) calcd C 74.45, H 10.13, found C 74.34, H 10.05. This compound (3.270 g, 5.48 mmol), K₂CO₃ (0.907 g, 6.56 mmol), and benzyl bromide (780 μ L, 6.57 mmol) were refluxed in DMF (30 mL) for 4 h. Et₂O (150 mL) was added, and the mixture was successively washed with water (50 mL) and saturated aqueous NaHCO₃ (50 mL). After drying of the organic layers over Na₂SO₄ and evaporation of the solvents, the residue was chromatographed (hexane/ethyl acetate 30:1 $\rightarrow$ 10:1), yielding compound **9** as a colorless oil (3.505 g, 93%): $[\alpha]_D^{20}$ -3.8° (c 2.3, CHCl₃); IR 3033, 2926, 2854, 1732, 1583, 1268, 835, 774, 696; ¹H NMR (CDCl₃, 200 MHz) δ 7.24–7.42 (m, 10H), 7.21 (m, 1H), 6.79 (m, 2H), 5.33 (s, 2H), 5.07 (s, 2H), 3.75 (m, 1H), 2.52 (m, 2H), 1.27–1.54 (br, 28H), 1.11 (d, J = 6.0, 3H), 0.88 (s, 9H), 0.04 (s, 6H); ¹³C NMR (CDCl₃, 50 MHz) δ 168.2, 155.4, 141.6, 138.1, 136.8, 135.7, 130.2, 128.6, 128.4, 128.2, 127.8, 127.1, 124.0, 121.8, 109.9, 70.4, 68.7, 66.9, 39.8, 33.5, 31.3, 29.7, 29.6, 29.4, 25.9, 25.8, 23.8, 18.2, -4.4, -4.7; MS m/z (rel intensity) 629 ([M - C₄H₉]⁺, 2), 611 (4), 585 (4), 567 (9), 521 (6), 493 (4), 431 (4), 417 (16), 181 (23), 91 (100); C₄₄H₆₆O₅Si (687.09) calcd C 76.92, H 9.68, found 76.85, H 9.58.

2-Benzyloxy-6-[(16*R*)-hydroxyheptadecyl]benzoic Acid Benzyl Ester (10**).** Compound **9** (3.500 g, 5.09 mmol) was treated with TBAF (1.0 M in THF, 7.0 mL, 7.00 mmol) for 15 h at ambient temperature. Water (20 mL) was added and

(25) For recent syntheses of other bioactive targets from this laboratory, see: (a) Fürstner, A.; Weintritt, H. *J. Am. Chem. Soc.* 1998, 120, 2817. (b) Fürstner, A.; Langemann, K. *J. Am. Chem. Soc.* 1997, 119, 9130. (c) Fürstner, A.; Müller, T. *J. Org. Chem.* 1998, 63, 424. (d) Fürstner, A.; Weintritt, H. *J. Am. Chem. Soc.* 1997, 119, 2944. (e) Fürstner, A.; Seidel, G. *J. Org. Chem.* 1997, 62, 2332. (f) Fürstner, A.; Langemann, K. *Synthesis* 1997, 792. (g) Fürstner, A.; Kindler, N. *Tetrahedron Lett.* 1996, 37, 7005. (h) Fürstner, A.; Langemann, K. *J. Org. Chem.* 1996, 61, 8746. (i) Fürstner, A.; Ernst, A.; Krause, H.; Ptock, A. *Tetrahedron* 1996, 52, 7329. (j) Fürstner, A.; Weintritt, H.; Hupperts, A. *J. Org. Chem.* 1995, 60, 6637.

- 1 -

4-ARYLMETHYLOXYMETHYL PIPERIDINES AS TACHYKININ ANTAGONISTS

5 ~~This invention relates to a class of azacyclic~~
~~compounds, which are useful as tachykinin antagonists.~~
More particularly, the compounds of the invention
comprise an azacyclic ring system substituted by an
arylmethyloxy or arylmethylthio moiety.

10 The tachykinins are a group of naturally-
occurring peptides found widely distributed throughout
mammalian tissues, both within the central nervous system
and in the peripheral nervous and circulatory systems.
The structures of three known mammalian tachykinins are
as follows:

15 Substance P:

Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH₂

Neurokinin A:

His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH₂

Neurokinin B:

20 Asp-Met-His-Asp-Phe-Phe-Val-Gly-Leu-Met-NH₂

Evidence for the usefulness of tachykinin
receptor antagonists in pain, headache, especially
migraine, Alzheimer's disease, multiple sclerosis,
attenuation of morphine withdrawal, cardiovascular
25 changes, oedema, such as oedema caused by thermal injury,
chronic inflammatory diseases such as rheumatoid
arthritis, asthma/bronchial hyperreactivity and other
respiratory diseases including allergic rhinitis,
inflammatory diseases of the gut including ulcerative
30 colitis and Crohn's disease, ocular injury and ocular
inflammatory diseases, proliferative vitreoretinopathy,
irritable bowel syndrome and disorders of bladder
function including cystitis and bladder detrusor hyper-
reflexia is reviewed in "Tachykinin Receptors and



02
 148
 146

Total Synthesis of Deacetyl-caloporoside, A Novel Inhibitor of The GABA_A Receptor Ion Channel

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Abstract : The total synthesis of deacetyl-caloporoside (1) which contains a β -D-mannopyranoside unit has been accomplished by coupling of the disaccharide-like segment derived from methyl α -D-mannopyranoside, with the hydroxyheptadecyl salicylic acid segment. The biological activity of 1 has also been evaluated.

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Deacetyl-caloporoside (1) is a novel fungal inhibitor of the binding of ^{35}S -labelled *t*-butylbicyclopophosphorothionate (^{35}S -TBPS) to the GABA_A/benzodiazepine chloride channel receptor complex *in vitro*.¹⁾ On the other hand, caloporoside (2) has been reported to inhibit phospholipase C.²⁾ It would be therefore important to examine the possibility that deacetyl-caloporoside (1) also shows a similar enzyme inhibitory activity.

Herein, we report the first total synthesis of deacetyl-caloporoside (1) by coupling of a disaccharide-like segment (8) with a hydroxyheptadecyl salicylic acid segment (18) as part of a ongoing program to clarify the mode of action of enzyme inhibitors.³⁻⁵⁾

The segment 8 was synthesized from methyl α -D-mannopyranoside through a common intermediate 4. Methyl α -D-mannopyranoside was *O*-benzylated (BnBr, NaH, DMF, 17h) and subsequently hydrolyzed [aq 0.5M H₂SO₄-AcOH (1 : 4), 100°C, 1.5h] to give the protected mannose 3 (71%, syrup), which reacted with 2-naphthalenethiol (BF₃-Et₂O, CH₂Cl₂, 12h) to afford the 1-thio-mannoside 4⁶⁾ (85%, syrup). On the other hand, NaBH₄ reduction (MeOH, 22h) of 3 followed by selective silylation (TBDPSCI, imidazole, DMF, 1.5h) gave the alcohol 5⁶⁾. In β -D-mannosylation of 5, a large number of methods^{7,8)} including glycosyl donors [phenyl-1-thio and (pyridine-2-yl)-1-thio-mannosides *etc.*] and solvents used were assayed; the selective formation of β -mannopyranosides was in general difficult. The best result was realized by modification of the conditions of Fraser-Reid⁹⁾ using the bulky glycosyl donor 4 and acceptor 5 in EtOAc (NIS, 0.15M TfOH in CH₂Cl₂, -40°C, 1.5h). This process gave, after de-*O*-silylation (TBAF, THF, 13h), the desired β -mannoside 6⁶⁾ as the major product [64%, syrup, $[\alpha]_D$ -22° (c 0.9, CHCl₃)] accompanied by the α -mannoside 7⁶⁾ [24%, syrup, $[\alpha]_D$ +28° (c 1.0, CHCl₃)]. The stereochemistry (β or α) of the mannosides 6 and 7 was determined by NMR studies, especially based on the coupling constants¹⁰⁾ of $1J(^{13}\text{CH})$ (157 and 168 Hz, respectively) in ¹³C NMR (CDCl₃). The structures of 6 and 7 were finally confirmed by the synthesis of 1. Oxidation of 6 with oxalyl chloride-DMSO (Et₃N, CH₂Cl₂, -78° → 0°C, 0.5h) gave the aldehyde, which was then treated with sodium chlorite (H₂NSO₃H, aq dioxane, 1h) to afford the corresponding carboxylic acid 8⁶⁾ [91%, syrup,

$[\alpha]_D -29^\circ$ (c 1.0, CHCl_3), FAB-MS(m/z) 1077(M-H^-). The α -mannoside **7** was similarly converted into the carboxylic acid **9**⁶ [89%, syrup, $[\alpha]_D +8.7^\circ$ (c 1.0, CHCl_3), FAB-MS(m/z) 1077(M-H^-)].

The other segment **18** was synthesized from the phosphonium salt **10**, which was prepared from (*R*)-1,3-butanediol in our laboratories.¹¹ The Wittig reaction (DMSO-NaH , *n*-BuLi, THF, 1h)¹¹ of **10** with the aldehyde **11**¹² gave the bromo-olefin **12**, which was treated with PPh_3 (MeCN, 80°C , 25h) to give the new phosphonium salt **13**⁶ (90%, syrup). The second Wittig reaction (DMSO-NaH , 1.5h) of **13** with the hemiacetal **14**¹³ afforded a mixture of olefins, which was submitted to catalytic reduction (H_2 , Pd-C, EtOH) to yield the saturated alcohol **15**⁶ [64%, mp $80\sim 81^\circ\text{C}$ (toluene), $[\alpha]_D -4.2^\circ$ (c 1.1, CHCl_3), FAB-MS(m/z) 405(M-H^-)]. De-*O*-methylation¹⁴ with LiCl (DMF, 150°C , 3h) provided a salicylic acid derivative **16**⁶ [87%, mp $77\sim 79^\circ\text{C}$ (toluene), $[\alpha]_D -3.8^\circ$ (c 1.1, MeOH)], the physico-chemical data being identical with those for the naturally derived sample.¹

Esterification of **16** (EtOAc-MeOH, 2h) with benzophenone hydrazone and HgO ¹⁵ (Petr. ether, 3h) gave the benzhydryl ester **17**⁶, which was selectively benzylated (BnBr, K_2CO_3 , Me₂CO, 15h) to the alcohol **18**⁶ [85%, syrup, $[\alpha]_D -2.7^\circ$ (c 1.1, CHCl_3), FAB-MS(m/z) 671(M+Na^+)].

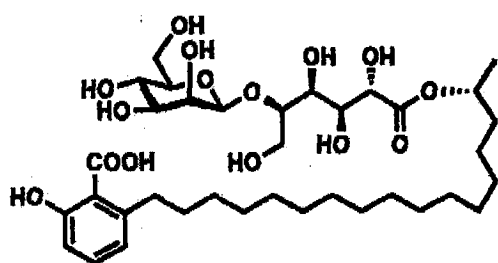
The final stage, coupling of the carboxylic acid **8** with the alcohol **18**, was accomplished by using the modified conditions of Yamaguchi.¹⁶ After treatment of **8** with 1-naphthoyl chloride (Et_3N , THF, 1h), the solution was filtered and the filtrates were added to the alcohol **18** (DMAP, PhMe, 1h) to give the ester **19**⁶ [74%, syrup, $[\alpha]_D -22^\circ$ (c 0.7, CHCl_3), FAB-MS(m/z) 1732(M+H+Na^+)]. Hydrogenolysis of **19** (H_2 , Pd-C, dioxane-aq AcOH) furnished the desired product **16**⁶ [72%, syrup, $[\alpha]_D -19^\circ$ (c 1.0, MeOH), FAB-MS(m/z) 731(M-H^-)], which was identical with natural deacetyl-caloporoside in all respects.¹ Similarly **9** was transformed into the α -mannoside analog **21**⁶ [58% total yield, syrup, $[\alpha]_D +18^\circ$ (c 1.0, MeOH), FAB-MS(m/z) 731(M-H^-)] through **20**⁶ [syrup, $[\alpha]_D +8.0^\circ$ (c 1.3, CHCl_3), FAB-MS(m/z) 1732(M+H+Na^+)]. Coupling of the acid **8** with the alcohol **17**, without protection of the phenolic hydroxyl group, gave the product **1** albeit in lower yield (20%) after hydrogenolysis.

On preliminary biological assays, both products **1** and **21** were found to exhibit a similar inhibitory activity against the binding of a ligand to the GABA_A/benzodiazepine chloride channel receptor complex *in vitro* (IC_{50} 60 - 40 μM), and also against phospholipase C isolated from rat brain (IC_{50} 12 μM). Further biological evaluation is underway and will be reported in due course.

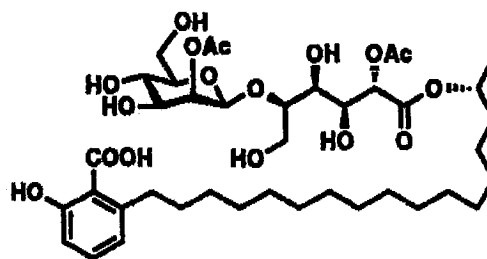
Acknowledgment: We are grateful to Meiji Seika Kaisha, Ltd., Shikoku Chemicals Co., Yamanouchi Pharmaceutical Co., Ltd. and the Institute of Microbial Chemistry for the generous support of our program.

REFERENCES AND NOTES

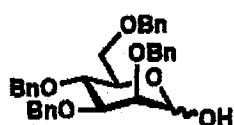
1. Shan, R.; Anke, H.; Nielsen, M.; Sterner, O.; Witt, M. R. *Nat. Prod. Lett.*, **4**, 171 (1994).
2. Weber, W.; Schu, P.; Anke, T.; Velten, R.; Steglich, W. *J. Antibiot.*, **47**, 1188 (1994).
3. Tatsuta, K.; Niwata, Y.; Toshima, K.; Nakata, M. *Tetrahedron Lett.*, **31**, 1171 (1990).
4. Tatsuta, K.; Kitagawa, M. *J. Antibiot.*, **47**, 389 (1994); *Heterocycles*, **38**, 1233 (1994).
5. Tatsuta, K.; Miura, S.; Ohta, S.; Gunji, H. *Tetrahedron Lett.*, **36**, 1085 (1995).
6. All compounds were purified by silica-gel column chromatography and/or recrystallization, and were fully characterized by spectroscopic means. Optical rotations were measured using a 0.5 dm tube at 22°C .

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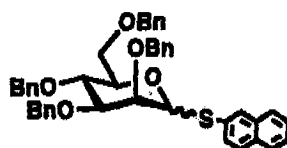
1: Deacetyl-caloporoside



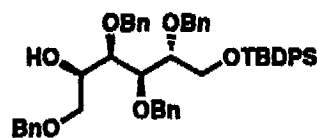
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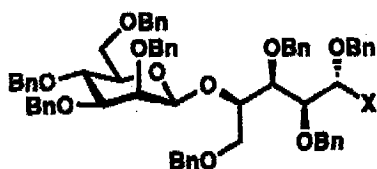
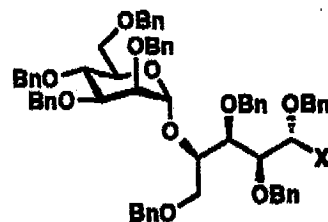
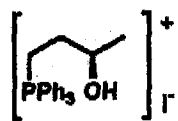
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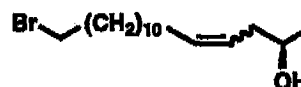
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6: X = CH₂OH
8: X = COOH7: X = CH₂OH
9: X = COOH

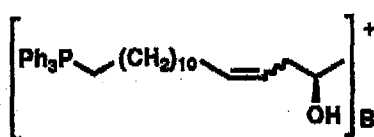
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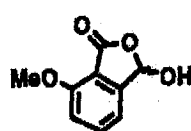
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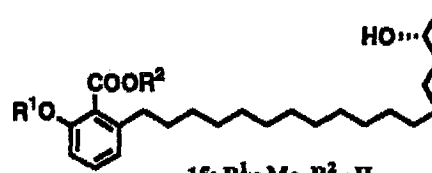
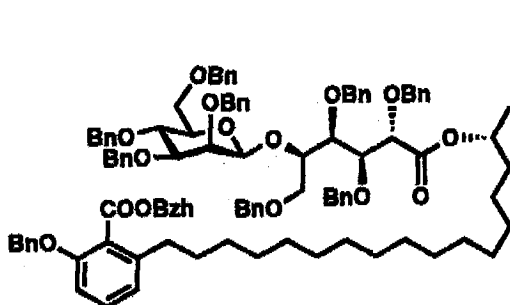
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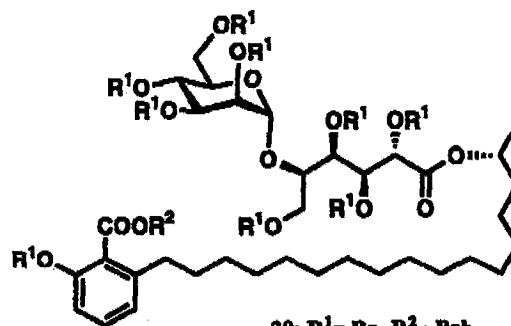
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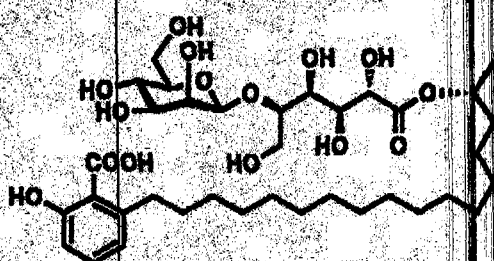


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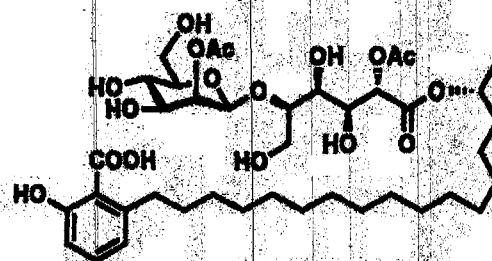
15: R¹ = Me, R² = H
16: R¹ = R² = H
17: R¹ = H, R² = Bzh
18: R¹ = Bn, R² = Bzh

19

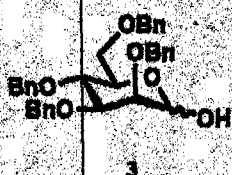
20: R¹ = Bn, R² = Bzh
21: R¹ = R² = H



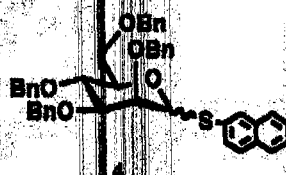
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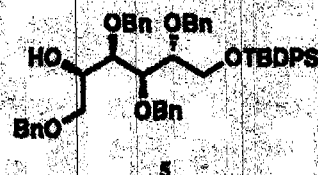
2: Caloporoside



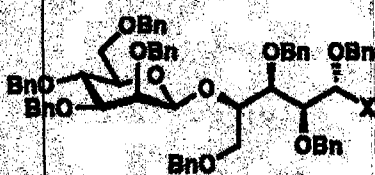
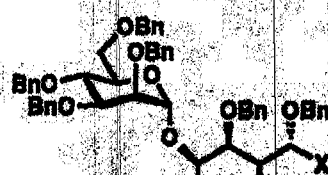
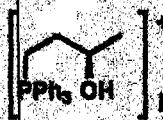
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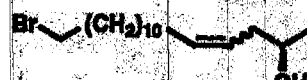
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6: X = CH₂OH
8: X = COOH7: X = CH₂OH
9: X = COOH

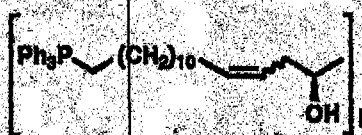
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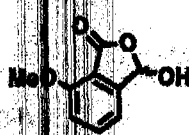
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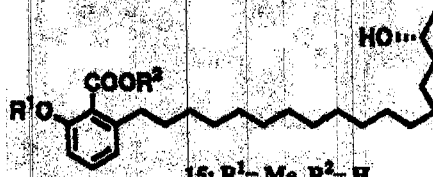
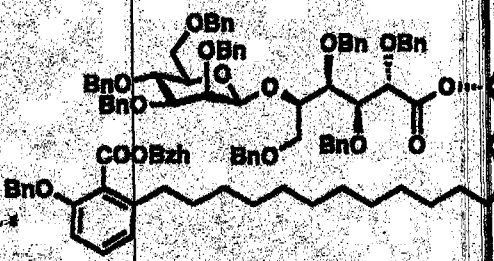
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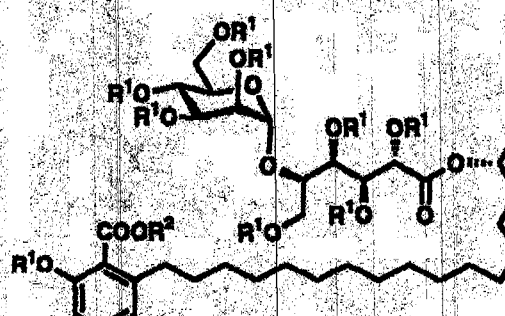
13



14

15: R¹ = Me, R² = H
16: R¹ = R² = H
17: R¹ = H, R² = Bzh
18: R¹ = Bn, R² = Bzh

19

20: R¹ = Bn, R² = Bzh
21: R¹ = R² = H

**SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
DIRECCION DE NUEVAS CREACIONES**

2020
Bogotá, D.C.,

Abogado:
LUIS PATIÑO LEYVA

ASUNTO:	Radicación No.	03 07 7712
	Trámite	011
	Evento	379
	Actuación	430
	Oficio	<u>018</u> / 2
	Folios	14

Como resultado del Examen de Fondo, realizado con fundamento en el artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se comunica:

Documentos estudiados:

Descripción: Folios 35 a 58 como se radicó inicialmente.

Reivindicaciones: Folios 59 a 61 como se radicó inicialmente.
Folios 136 a 137 como se fue modificado.

Prioridad: DE 101 11 682.9 (09/03/2001)

Objeto de la Invención

Título de la invención: "Derivados de caloporósido, procedimiento para repararlos"

Problema Técnico Planteado: "Necesidad de nuevos compuestos quimioterapéuticos capaces de alterar el ciclo celular al inhibir las quinasas dependientes de ciclinas"

Solución: "Proveer compuestos activos producidos por la cepa *Gloeoporus dichrous*"

IPC: A61K 31/70; C07H 13/06

Excepciones a la Patentabilidad (Art 20 D 486 literal c)

Aunque los microorganismos se consideran una excepción a la prohibición de la patentabilidad, esta excepción es válida siempre y cuando el microorganismo

que se desea proteger no sea un simple aislamiento de la naturaleza sino que se trata de un microorganismo mutante o que en general posea una característica funcional que lo diferencia de la forma natural del mismo.

Por lo tanto, la materia reclamada en la reivindicación 12 que trata del microorganismo *Gloeoporus dichrous*, sin ninguna característica funcional que defina la cepa no se considera un material patentable.

Análisis de la Solicitud de Patente

Reivindicaciones (Art 30 D 486, Art 22 PCT)

Claridad y concisión:

La reivindicación 8 dice:

"Procedimiento para la preparación de un compuesto de fórmula I o de una sal de el fisiológicamente tolerada de acuerdo con una o varias de las reivindicaciones 1 a 7, caracterizado por que el microorganismo *Gloeoporus dichrous* (Fr:Fr) Bres. ST001714, DSM 13784 o de una de sus variantes o mutantes se fermentan bajo condiciones apropiadas, uno o varios derivados de caloporósido se aíslan y eventualmente ellos son convertidos en sales fisiológicamente toleradas", con respecto a la cual se anota lo siguiente:

- ✓ Existen problemas de redacción, se da a entender que el proceso esta caracterizado por que el microorganismo se fermenta, lo cual técnicamente no es correcto.
- ✓ Presenta que la fermentación ocurre "bajo condiciones apropiadas", lo que no es objetivo y no define las condiciones particulares del procedimiento.

La reivindicación 11 dice:

"Procedimiento para la preparación de medicamentos de acuerdo con la reivindicación 11, caracterizado por que al menos un..." lo que demuestra una falsa dependencia de si misma.

La reivindicación 9 presenta el mismo inconveniente, ya que esta depende de si misma.

Soporte en la descripción:

La reivindicación 1 define R1, R2, y R3 como hidrógeno o radicales acilo de 2 a 10 átomos de carbono, donde el acilo puede ser una cadena lineal, ramificada, saturados, monoinsaturados o diinsaturados, y define a R4 como hidrógeno o $-C(O)(CH_2)_nCOOH$ y a n como 7 y en la parte experimental solo se muestran caloporósidos B, C, D, E y F. Se sugiere limitar el alcance de la definición de los radicales para la molécula.

La reivindicación 8 no está soportada en cuanto a las variantes y mutantes de la cepa de *Gloeoporus dichrous*, por que sólo (folio 41, párrafo 2) presenta la forma general en la cual se puede obtener un mutante de una cepa, pero no aporta ejemplos o datos que prueben que la invención ha sido desarrollada por una cepa mutante.

Determinación del Estado de la Técnica

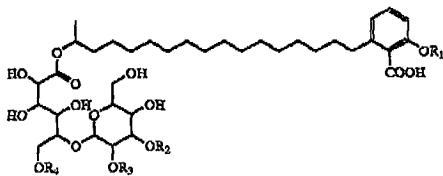
El Estado de la Técnica se determinó con base en la fecha de presentación 08-03-2001 y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Item	Documento	Título	Fecha de Publicación
D1	J.Org.Chem 1998.63.3072-3080	Total synthesis of Caloporoside	16.04.1998
D4	Tetrahedron letters Vol 37, No 14, pp 2453-2456	Total Synthesis Of Deacetyl-Caloporoside, A Novel Inhibitor Of The GABA A Receptor Ion Channel	1996

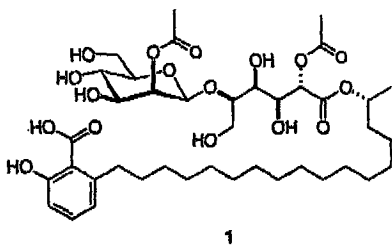
Análisis de Patentabilidad (Art 14 D486)

Nivel Inventivo (Art18 D486)

La reivindicación 1 dice: "Un compuesto de fórmula I en la cual R1, R2 y R3 independientemente uno del otro son hidrógeno o radicales acilo que tienen 2-10 átomos de carbono; y R4 es hidrógeno o -C(O)(CH2)nCOOH, en donde n es 1 a 7; con la excepción de que R1, R2, R3 y R4 no son todos H; y sus sales fisiológicamente toleradas."



D1 enseña el compuesto caloporósido como un inhibidor potente y selectivo de la actividad de fosfolipasa C y su análogo deacilado (página 3073, figura 2); así como también una ruta sintética que incluye la protección de ciertos sitios de la molécula con grupos como OAc en la posición de R3 según la presente solicitud (página 3073, columna 2, figura a la derecha).



D1 también presenta que el caloporósido es un producto de el microorganismo *Caloporous dichrous*,

El compuesto de la presente solicitud se diferencia de D1 en que R1, R2, R3 y R4 no son todos H y que la manosa no posee grupos sustituyentes en D1.

El Efecto Técnico que logra la diferencia es la actividad inhibitoria específica sobre CDK-4.

El Problema Técnico Objetivo de esta solicitud es la necesidad de proveer compuestos alternativos para inhibir CDK-4

D4 describe la obtención del análogo deacetilado del caloporósido el cual posee actividad inhibitoria del caloporósido sobre la fosfolipasa C (página 2453, párrafo 1) y las formas de proteger la manosa y el resto de la molécula por medio de un grupo bencilo (pagina 2454 y 2455).

Para el experto, D4 sugiere grupos sustituyentes sobre el azúcar de la molécula lo cual sugería al experto a incorporar sustituyentes en dicha posición en los

compuestos caloporósidos ya revelados por el estado del arte como D1, para llegar a los compuestos mostrados por la presente solicitud.

Se concluye que la materia reclamada en la reivindicación 1 y sus dependientes carecen de nivel inventivo.

Conclusión

Los requisitos de Patentabilidad de la presente Solicitud se encuentran afectados así:

	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	J.Org.Chem 1998.63.3072-3080	1-7, 11	Nivel inventivo
D4	Tetrahedron letters Vol 37, No 14, pp 2453-2456	1-7, 11	Nivel inventivo

Respuesta a los argumentos del solicitante:

El Señor solicitante argumenta que en relación a la formula I de la presente invención, los grupos R1 a R4 no pueden representar H y D2 solo menciona derivados de caloporósido en donde R1-R4 son H, por lo que no destruye la novedad de los derivados de caloporósido enseñados por la presente invención.

A este respecto, este despacho reconoce que los compuestos presentados por D2, corresponden a deacetylcaloporósido o caloporósido, que no corresponden a los mismos compuestos reclamados por la presente solicitud.

El Señor solicitante menciona que no tiene en cuenta D1 ni D3 para realizar la evaluación de nivel inventivo dadas las fechas de publicación y la difícil consecución de las mismas anterioridades. Afirma que Faenza. Et al. J, Biol. Chem 2000, 275, 30520-30524 muestra: que la inhibición de fosfolipasa C influencia la proliferación celular, que con un aumento en la expresión de fosfolipasa C hay un incremento de CDK4, pero no que CDK4 sea inhibida por caloporósido y ni que existe un efecto antiproliferativo resultante de la inhibición de CDK4 mencionada por dicha referencia.

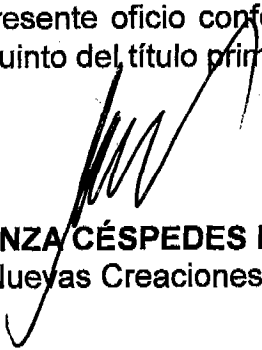
A lo anterior, se aclara que en el concepto técnico 569 se identificó a D1 como J.Org.Chem.1998, 63, 3072, cuya publicación fue hecha en Abril 16 de 1998. Se acepta que D3 no sea tenida en cuenta para el análisis.

Se anota que en el estado del arte estos compuestos habían sido reconocidos por ser inhibidores fuertes y selectivos de fosfolipasa C (Total synthesis of deacetylcaloporoside, A novel Inhibitor of the -gaba Receptor Ion Channel, página 1, párrafo 1), esto junto con lo sugerido por Faenza, cuando dice que la inhibición de la enzima fosfolipasa C altera el ciclo celular, motivaría al experto a pensar que el caloporósido y sus derivados tienen una actividad inhibitoria de enzimas que tienen acción sobre el ciclo celular, resultando en una acción citotóxica.

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En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de 60 días contados a partir de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única N° 10 de 2001.


ALIX CARMENZA CÉSPEDES DE VERGEL
Directora de Nuevas Creaciones

El anterior requerimiento fue notificado por fijación en lista, de fecha
12 FEB. 2008

CONCEPTO TECNICO NUMERO: 2959	EXAMINADOR TÉCNICO Código:202092
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